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# IMPACT OF WET DISTILLERS GRAINS PLUS SOLUBLES AND ANTIOXIDANTS ON A BASIC MECHANISM OF BEEF TENDERIZATION

Michael D. Chao
*University of Nebraska-Lincoln*, yak0917@yahoo.com

# IMPACT OF WET DISTILLERS GRAINS PLUS SOLUBLES AND ANTIOXIDANTS ON A BASIC MECHANISM OF BEEF TENDERIZATION

by

Michael Chao

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# IMPACT OF WET DISTILLERS GRAINS PLUS SOLUBLES AND ANTIOXIDANTS ON A BASIC MECHANISM OF BEEF TENDERIZATION

Michael Chao, Ph.D.

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Adviser: Chris R. Calkins

Feeding high levels of wet distillers grains plus solubles (WDGS) increases polyunsaturated fatty acid (PUFA) levels in beef. Perhaps, WDGS in feedlot diets increases PUFA concentration in the SR membrane, thereby altering membrane integrity, resulting in more rapid calcium leakage and improved tenderness. Feeding antioxidants may mitigate such effects. In the first study, effects of feeding 50 % WDGS on SR membrane composition, free calcium concentration and tenderness were studied. The SR membrane from steers fed WDGS were more tender, had higher free calcium concentration, had more PUFA, more phosphatidylcholine (PC), less phosphatidylethanolamine (PE) and less total phospholipids when compared to SR membrane from steers fed corn-only ($P < 0.05$). In the second study, effects of feeding 30% WDGS and two antioxidants on muscle tissue fatty acid profiles, color and lipid oxidation of beef were studied. Steaks from cattle fed WDGS had increased muscle tissue PUFA, were not different in color, and were less oxidized when compared to steaks from steers fed corn-only ($P < 0.05$). Supplementing vitamin E (E) was very effective in reducing discoloration and lipid oxidation, and increasing muscle tissue E levels compared to the diets without E supplementation ($P < 0.05$). Feeding Agrado Plus (AG)

had minimal effect of discoloration and lipid oxidation, but significantly increased muscle tissue ethoxyquin level compared to diets without AG supplementation. In the third study, effects of feeding WDGS and the two antioxidants on SR membrane composition and proteolysis were studied. Again, feeding WDGS increased total PUFA and PC, but decreased PE in the SR membrane ($P < 0.05$). Conversely, E supplementation in WDGS diet prevented such shifts, while supplementing AG in the corn-only diet created similar effects as the WDGS-only diet for SR membrane phospholipid profile. At 2 d postmortem, steaks from steers fed WDGS had more troponin-T degradation compared to steaks from steers fed corn-only or either diet supplemented with E only ($P < 0.05$). Feeding WDGS induced SR membrane phospholipid shift and clearly contributed to cattle SR membrane instability, while E supplementation may prevent such phenomenon.

**Key words**: Beef, distillers grains, antioxidants, sarcoplasmic reticulum.

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# INTRODUCTION

This study seeks to understand the effect of distillers grains diets on post-harvest changes in beef that may improve tenderness. In an effort to support energy independence, the U.S. has sought to promote production of ethanol as a source of biofuel. Most ethanol production in the U.S. uses corn as the starch source because corn is the most readily accessible grain source in this country (NCGA, 2013). Distillers grains are the major by-product of ethanol production. During ethanol production, corn kernels are ground, and the starch is fermented by yeast to produce alcohol. The whole stillage is then centrifuged to remove the coarser grain particles. The leftover solubles fraction can be dried with the coarse grain particles to produce dried distillers grains plus solubles (DDGS) or added back to the coarse grain particles without drying to produce wet distillers grains plus solubles (WDGS) (Stock et al., 2000).

When compared to corn, distillers grains are not only less expensive, but also contain up to three times the levels of protein, fiber, and fat (Klopfenstein et al., 2007). Hence, WDGS have been used widely in feedlot diets at levels varying from 10 to 80% on a dry matter basis. Mello et al. (2012) showed that beef from steers fed 30% of WDGS had increased proportions of polyunsaturated fatty acids (PUFA), linoleic, and trans fatty acids, but decreased proportions of monounsaturated fatty acids (MUFA), when compared to beef from steers fed corn.

Although many studies (Koger et al., 2010; Mello et al., 2012; Roeber et al., 2005) on beef quality from feeding distillers grains showed no differences in tenderness, a recent study from our laboratory (Senaratne, 2012) revealed an intriguing phenomenon. Beef from steers fed WDGS was more tender than beef from steers fed corn, and beef

from steers fed antioxidants was tougher than beef from steers that were not fed antioxidants. This is not the first time that feeding WDGS has been reported to improve tenderness, nor that cattle fed antioxidants have been found to be less tender. Depenbusch et al. (2009) reported sensory overall tenderness ratings increased linearly as dietary level of distillers grains increased from 0 to 75%, and Segers et al. (2011) showed that beef from cattle supplemented with 25 % DDGS for 100 d was more tender compared to beef from cattle supplemented with soybean meal. In addition, Secrist et al. (1995) showed that vitamin E supplementation decreased tenderness. There is no study to the author's knowledge that explains these phenomena.

The working hypothesis is that the overall increase in PUFA from WDGS causes an increase in PUFA of sarcoplasmic reticulum (SR) membrane, the organelle that sequesters calcium within the muscle cell. This could contribute to instability of the SR membrane, making it more susceptible to oxidation and thereby causing the early post-rigor release of calcium. Early calcium release would more quickly activate calcium-dependent proteolytic enzymes like calpains, which tenderize beef during aging, and thus improve beef tenderness.

The objectives of the research were to understand the tenderization mechanism in beef from cattle fed a high concentration of WDGS. More specifically, the studies aimed to complete the following objectives:

1) Characterize the relationships between PUFA content and lipid oxidation on calcium release during postmortem storage of beef longissimus muscle;

2) Determine the time course of calcium release and subsequent proteolysis of meat;

3) Quantify PUFA and phospholipid changes in the membrane of the SR from feeding

WDGS;

4) Assess the effects of oxidation on beef longissimus muscle tenderness; and

5) Evaluate feeding of antioxidants to optimize beef tenderness using knowledge of this tenderization mechanism.

The long-term goal of this research is to improve the tenderness of U.S. beef by better understanding the biology of beef tenderization. U.S. beef enjoys a strong standing in the global marketplace. This research could provide the basis for development of strategies to optimize tenderness and achieve the highest possible value in both domestic and international market.

# LITERATURE REVIEW

## Sarcoplasmic reticulum (SR): The calcium reservoir

Calcium acts as the intracellular signal for contraction in living muscle. Consequently, control of calcium is highly regulated. The intracellular calcium pool is predominately sequestered within the SR. When an action potential is triggered through neural activity, the SR is stimulated to release calcium into the sarcoplasm where its presence facilitates interaction of actin and myosin, thereby causing contraction. When the neural signal dissipates and with the presence of ATP, calcium is actively sequestered back into the SR, resulting in muscle relaxation (Aberle et al., 2001).

After death, the depletion of oxygen causes a decline in ATP production which ultimately allows calcium to seep out of the SR through calcium release channels (Greaser et al., 1969). With the calcium pumps losing their ability to uptake calcium, there is an increase in sarcoplasmic calcium as rigor mortis develops (Jeacocke, 1993). Further release of calcium occurs during extended post-rigor storage either through the calcium release channels (Hidalgo et al., 2000) or channels formed by the degradation of SR membrane phospholipids (Ji and Takahashi, 2006). The presence of free sarcoplasmic calcium facilitates activation of the calpain enzyme system with subsequent proteolysis of muscle proteins and a consequent improvement in meat tenderness (Koohmaraie, 1994, 1992; Taylor and Koohmaraie, 1998). This research is focused on understanding how changes in SR membrane composition can affect the release of free calcium. Therefore, it is vital to fully understand SR structure and protein functions to interpret research results at this level.

**Transverse-tubule (T-tubule) and SR structures.** The T-tubule and SR are membrane-bound organelles found between myofibrils. The network of T-tubule and SR forms a mesh covering the myofibrils. Although the T-tubule is technically not part of the SR, the close proximity of the T-tubule to the SR allows for signal transduction between these respective structures. T-tubules are invaginations of the sarcolemma, and a section of T-tubule forms a triad with two sections of the junctional SR (also known as terminal cisternae) between the A and I bands of the sarcomere (Du and McCormick, 2009). The structure of T-tubules allows quick distribution of the action potential from the sarcolemma for quick calcium release. The SR itself can be divided into the enlarged junctional SR, where it is in close proximity with the T-tubules, and the thinner longitudinal SR, where it is more meandering and runs along the length of the sarcomere (Sorrentino, 2011).

**Type of SR proteins.** The balance of calcium storage, release and uptake is achieved through the concerted action of three major classes of calcium-regulatory proteins: (1) luminal calcium-binding proteins such as calsequestrin for calcium storage; (2) SR calcium release channels such as type 1 ryanodine receptors (RyR1) for calcium release; and (3) sarco-endoplasmic reticulum calcium-ATPase (SERCA) pumps for calcium reuptake (Rossi and Dirksen, 2006).

**Luminal calcium-binding proteins.** A number of different SR luminal calcium-binding proteins participate in calcium storage to help maintaining luminal calcium at around 1 mM (Fryer and Stephenson, 1996). Calsequestrin is the most abundant luminal calcium-

binding protein, which it accounts for approximately 27% of all junctional SR proteins (Costello et al., 1986). Approximately one-third of calsequestrin amino acids are acidic, and these acidic amino acids are important for calcium binding. Rather than the presence of discrete calcium-binding sites, acidic amino acids create a net negative charge to enhance calcium binding (Wang et al., 1998). Furthermore, calsequestrin is capable of high-capacity (~40 mol calcium/mol protein) but low-affinity ($K_d$ = 40 μM) calcium binding, for strong buffering capacity and quick release of calcium when an action potential arrives (MacLennan and Wong, 1971).

Other than calsequestrin, histidine-rich calcium-binding protein and junctate are the other two significant luminal calcium-storage proteins located in the junctional SR. Although they all have their own unique role for calcium-binding, it is safe to say all three calcium-binding proteins serve to significantly enhance the level of high-capacity calcium storage (Rossi and Dirksen, 2006). In addition, all three proteins are anchored to the junctional SR membrane through interactions with junctin (not to confuse with junctate, junctin is a calcium sensor while junctate is a storage protein), triadin, and RyR1 (Zhang et al., 1997). By binding to both RyR1 and the luminal calcium-storage proteins, triadin and junctin can relay information regarding luminal calcium store content to RyR1 (Beard et al., 2005).

Sarcalumenin (SAR) is a high-capacity (~35 mol calcium/mol protein), moderate-affinity ($K_d$=300 μM) is yet another calcium-binding protein of significance that is primarily located in the longitudinal SR (Leberer et al., 1990). Because of the location of SAR, it is possible that this protein also interacts with SERCA. Research results from SAR knockout mice has shown that SAR not only contributes to calcium buffering within

the longitudinal SR, but may also influence calcium uptake (Yoshida et al., 2005).

**Calcium release channels.** The RyR1 located in the junctional SR is the primary channel of SR calcium release during skeletal muscle contraction. Such calcium release is controlled by a unique interaction between the dihydropyridine receptors (DHPR) located on the T-tubule membrane and RyR1 located in the junctional SR. The DHPR functions as a voltage sensor for RyR1. When the action potential spreads down the T-tubule, membrane depolarization causes conformational changes of the DHPR, which activates and initiates calcium release through nearby RyR1 (Fill and Copello, 2002).

Ryanodine receptors (RyR) were first identified based on their high-affinity binding to the plant alkaloid ryanodine (Campbell et al., 1987). Three RyR isoforms have been identified, and RyR1 is the primary RyR expressed in skeletal muscle. The electrophysiological characterization of RyR1 reveals that RyR1 has poor ionic selectivity (low selectivity for calcium ions), but high conductivity. Although calcium has to compete with magnesium, sodium and potassium, the high conductivity still allows rapid calcium release during muscle contraction (Fill and Copello, 2002; Lindsay et al., 1991). The C-terminal one-fifth of RyR1 is anchored on the junctional SR membrane, while the N-terminal four-fifths of the protein is located in the junction between the SR and T-tubule and interacts with the DHPR and a variety of other junctional regulatory proteins (Du et al., 2002).

The RyR1channel activity is regulated by both sarcoplasmic and luminal calcium levels. Low sarcoplasmic calcium concentration (1–10 μM) activates calcium release, whereas high sarcoplasmic calcium concentration (1–10 mM) inhibits calcium release

(Copello et al., 1997). On the other hand, luminal calcium levels are involved in conformational coupling through a complex interaction among calsequestrin, junctate, triadin and RyR1 that indirectly affect RyR1 activity (Beard et al., 2005; Beard et al., 2002). At physiological levels of luminal calcium (~1 mM), the binding of calsequestrin to the triadin–junctin–RyR1 complex inhibits calcium release. However, a high level of luminal calcium (~4 mM) promotes a conformational change in calsequestrin that weakens its interaction with triadin and junctin, thus relieving the inhibitory effect on calcium release (Györke et al., 2004).

Other than calcium levels, a number of other molecules (magnesium and ATP) and associated proteins (homer, FK506 binding protein-12 and calmodulin) also regulate the activity of RyR1. Sarcoplasmic ATP stimulates whereas sarcoplasmic magnesium inhibits RyR1 activity (Copello et al., 2002; Smith et al., 1985). Both the short and long form of homer proteins activate RyR1 calcium release and are potent modulators of ryanodine binding to membranes enriched with RyR1 (Ward et al., 2004). FK506 binding protein-12 (FKBP12) binds to each protomer of RyR1 tetramer and profoundly enhances the activity of RyR1 (Avila and Dirksen, 2005). Avila et al. (2003) showed that mice with mutated RyR1 (which lack FKBP12 binding sites) released only half of the voltage-gated calcium in comparison to the controls, suggesting that FKBP12 plays an important role in muscle contraction and relaxation. Calmodulin activates the skeletal muscle calcium-release channel at low (nM) sarcoplasmic calcium concentrations and inhibits the channel at high (mM) sarcoplasmic calcium concentrations (Tripathy et al., 1995).

Finally, RyR1 activity is also regulated by a variety of post-translational modifications including oxidation and phosphorylation. Although SR redox potential

does not directly alter the amount of calcium released during normal muscle contraction (Rossi and Dirksen, 2006), it is particularly important postmortem. The RyR1 possesses a number of highly reactive sulfhydryl (SH) groups that are susceptible to oxidation (Sun et al., 2001). A couple of investigators have reported inclusion of pro-oxidants such as heavy metals oxidized SH groups near the calcium release site and induced rapid calcium release from loaded SR vesicles, while the addition of a reducing agent reversed the effect (Abramson and Salama, 1988; Trimm et al., 1986). Hidalgo et al. (2000) showed that oxidized RyR1 becomes active at low (μM) luminal calcium concentrations and was not inhibited by high (mM) sarcoplasmic calcium concentrations. Furthermore, oxidized RyR1 was no longer inhibited by magnesium, and subsequent reduction reversed these effects (Hidalgo et al., 2000). These results suggested that the oxidation of RyR1 postmortem might induce calcium release. On the other hand, RyR1 is also known to be phosphorylated by protein kinase A (PKA) and calmodulin kinase II (CaMKII) (Rossi and Dirksen, 2006). However, the effects of these proteinases are still highly controversial. The PKA phosphorylation has been found to activate (Gechtman et al., 1991), inactivate (Wang and Best, 1992), or have no effect (Chu et al., 1990) on RyR1 activity. Similarly, contradictory effects of CaMKII phosphorylation on RyR1 channel activity have also been reported (Dulhunty et al., 2001; Hain et al., 1994).

Other than RyR1, the inositol trisphosphate (IP3) receptor pathway is the other major calcium release pathway. Unlike RyR1, the IP3 receptors contribute to long-lasting elevations in sarcoplasmic calcium that regulate calcium-dependent gene transcription (Cárdenas et al., 2004). Defined by its name, the IP3 receptors are activated by IP3 and calcium, and the IP3 is generated by the hydrolysis of phospholipid phosphatidylinositol

(PI) by phospholipase C (PLC) (Michell, 1975). Many guanine nucleotide-binding proteins or tyrosine kinase-linked receptor activators can induce activity in PLC isozymes PLC-β and PLC-γ, which results in the cleavage of phosphatidylinositol into IP3 and diacylglycerol (Kasri et al., 2004; Yang et al., 2002). Although still up for debate, it has been speculated that the initially released calcium stimulates turnover of membrane phospholipids, which increase the production of IP3 from hydrolysis of phosphatidylinositol, thus inducing the release of more calcium from IP3 receptors (MacMillan et al., 2005; Taylor and Tovey, 2010).

**Calcium uptake.** Removal of calcium from the sarcoplasm following contraction primarily involves calcium uptake into the SR by SERCA. Although plasma membrane-located calcium-ATPase pumps and the sodium/calcium exchanger also play roles in calcium influx, SERCA-mediated calcium uptake is mainly responsible for maintaining a 10,000-fold calcium gradient across the SR membrane (Rossi and Dirksen, 2006). SERCA is a member of the family of P-type calcium-ATPases, which means these calcium transport proteins have to undergo phosphorylation in order to support calcium uptake (Apell, 2003).

There are five primary isoforms of SERCA encoded by three separate genes (Brandl et al., 1987). Since SERCA1a is the principal form of SERCA to SR calcium uptake in adult skeletal muscle, only its structure and function will be discussed in this section. SERCA1a is a monomeric protein located in the membrane of both junctional and longitudinal SR, with the bulk of the protein, including the N- and C-termini, located in the sarcoplasm (Toyoshima et al., 2000). The structure can be divided into three parts:

1) a large sarcoplasmic headpiece; 2) an anchored SR transmembrane region; and 3) a stalk domain that connects the sarcoplasmic headpiece to the transmembrane region (Toyoshima et al., 2000). The headpiece facilitates engagement of the gating mechanism and thereby regulates calcium binding and release (Toyoshima and Inesi, 2004). On the other hand, the transmembrane region of the protein contains two closely spaced-calcium binding sites. Two calcium ions bind with high affinity to the sites on the sarcoplasmic side of the protein and are released to the luminal side of the protein following ATP hydrolysis. For each ATP molecule hydrolyzed per reaction cycle, two calcium ions from the sarcoplasm are pumped into the SR lumen in exchange for two or three hydrogen ions (Yu et al., 1993). Furthermore, phospholipid phosphatidylethanolamine (PE) plays an important role in the function of SERCA. PE is bound to the transmembrane helices of the SERCA, and is replaced by calcium when calcium binds to the protein. Hunter et al. (1999) showed that the activity of the SERCA progressively increases when increasing amounts of PE are incorporated into reconstituted SR vesicles.

Sarcolipin is a low-molecular weight regulatory protein that co-purifies with SERCA1a, and it is thought to inhibit SERCA1a activity by decreasing its affinity for calcium, thereby lowering its $V_{max}$ (MacLennan et al., 1973; Odermatt et al., 1998). Furthermore, SERCA1a is inhibited by nitric oxide, and its calcium affinity decreases as pH is reduced (Ishii et al., 1998). Finally, SERCA1a function may also be regulated by the luminal calcium-binding protein, sarcalumenin (SAR). Specifically, SAR interacts with SERCA1a, and SR vesicles isolated from skeletal muscle of SAR-deficient mice exhibit decreased calcium accumulation due to a reduction in SERCA protein expression. However, SERCA mRNA levels in skeletal muscle are similar in wild-type and SAR-

deficient mice. The interaction of SAR with SERCA may serve to increase protein stability and reduce SERCA degradation (Yoshida et al., 2005). Thus, in the absence of SAR, a reduction in SERCA1a protein expression results in a decrease in SR calcium uptake.

**SR protein oxidation may induce rapid calcium release postmortem.** SR functionality is closely linked to the integrity of SR proteins. Free radicals formed in mitochondria can induce SR protein oxidation/degradation (Grubbs et al., 2013). There is very little information available on SR protein oxidation/degradation induced calcium release; however, it only makes sense that protein oxidation/degradation occurs as soon as antioxidant sources are depleted. As proteins are oxidized/degraded, they change their molecular structures and form intra/inter cross-links that alter their functions (Butterfield et al., 2006). It has been shown that oxidized SH groups can stimulate calcium release by forming a disulfide bond, which cause the RyR1 to maintain the open state (Abramson and Salama, 1989). Further research is needed to explore the relevant calcium release rates from SR protein oxidation and degradation. The oxidation/degradation of luminal proteins, especially calsequestrin, can result in adverse effects on SR storage ability, while early oxidation/degradation of SERCA may lead to calcium accumulation in the sarcoplasm pre-rigor and early post-rigor.

## Lipid oxidation of muscle food

Muscle has a complex physical structure and chemical composition that is very susceptible to postmortem oxidation (Wood et al., 2008). Reactive oxygen species (ROS)

produced in postmortem muscle such as hydroxyl radicals, peroxyl radicals, superoxide anions, hydrogen peroxides and nitric oxides can extensively oxidize meat components (mainly lipids) and eventually change the chemical and physical structure of meat (Burton and Traber, 1990; Butterfield et al., 2006). Most types of oxidation consist of three phases: 1) initiation - formation of free radicals; 2) propagation - the free-radical chain reactions; and 3) termination - the formation of nonradical products (Frankel, 2005; Lund et al., 2011). Those ROS can be formed within muscle due to intrinsic (metabolic functions) or extrinsic (processing) factors. In living muscle cells, mechanisms are available to prevent formation of ROS. However, after rigor mortis is complete, free radical accumulation increases within cells due to the collapse of intrinsic ROS-preventive mechanisms (Lobo et al., 2010).

Many studies (Koger et al., 2010; Mello et al., 2012; Senaratne et al., 2011; Sherbeck et al., 1995) have shown that feeding distillers grains to cattle significantly increases discoloration and contributes to off-flavor development of retail-displayed beef. It is well established that beef with higher PUFA concentrations are more likely to have increased myoglobin and lipid oxidation. However, a few recent studies documented contrasting results. Song et al. (2013) found that lipid oxidation values were not different between pigs fed 30% dried DDGS or corn-soybean-based control diets. Domenech et al. (2014) also found that steaks from steers fed corn only had higher lipid oxidation values and similar level of discoloration compared to steaks from steers fed WDGS. There is no doubt feeding distillers grains increases PUFA concentration in meat; however, for unknown reasons not all livestock respond in the same way to diets containing distillers grains.

Ladikos and Lougovois (1990) and Min and Ahn (2005) extensively discussed that lipid oxidation capacity in meats may depend on phospholipid composition, level of PUFA and antioxidant status. Since the plasma membranes consist of phospholipids with high levels of PUFA, a review of membrane lipid oxidation would be pertinent to the hypothesis being studied.

**Membrane structure.** The main purpose of membranes is to serve as barriers between two aqueous compartments with the ability to selectively transport molecules and conduct cell-to-cell communication (Cooper, 2000). The Fluid-Mosaic Model of biological membrane structure was first introduced in 1972, and it is still considered to be the most realistic membrane model available (Nicolson, 2014). The Fluid-Mosaic Model depicts biological membranes as a matrix made up of a mostly phospholipid bilayer with globular integral membrane proteins and glycoproteins that are inserted into the bilayer (Singer and Nicolson, 1972). The phospholipid bilayers are structured in a way that the exterior hydrophilic heads face the aqueous phase at each surface of the bilayer, and the interior is occupied by hydrophobic fatty acid chains, making the membrane impermeable to water-soluble molecules, including ions and most biological molecules (Harder et al., 1998). Given its name, the Fluid-Mosaic Model described membranes as "fluid", meaning the components such as the proteins and fatty acids can diffuse laterally in the plane of the membrane (Stanley, 1991). This is caused by the unsaturation of phospholipid fatty acids. The fatty acids of most phospholipids have one or more double bonds that introduce kinks into the hydrocarbon chains and make them difficult to pack together (Wood et al., 2008), while saturated fatty acids will decrease fluidity because of

the tight packing of these straight chain lipids (Cooper, 2000).

In phospholipids, one of the glycerol hydroxyls is esterified to a phosphate-containing polar group and an alcohol, and the other two are linked to long chain fatty acids, usually unsaturated (Stanley, 1991). In triacylglycerol, all three glycerol hydroxyls are connected to long/short chain saturated/ unsaturated fatty acids (Xu, 2000). Depending on the type of the phosphate binding on the alcohol, phospholipids can function as fuels, membrane structural elements, signaling agents, and/or surfactants (Tristram-Nagle and Nagle, 2004). The plasma membranes of animal cells contain four major phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SP), which together account for more than half of the lipid in most membranes (Cooper, 2000). The outer leaflet of the plasma membrane consists mainly of PC and SP, whereas PE and PS are the predominant phospholipids of the inner leaflet. A fifth phospholipid, phosphatidylinositol (PI), is also localized to the inner half of the plasma membrane (Alberts et al., 2002). Although PI is a quantitatively minor membrane component, it plays an important role in IP3 receptor activation (MacMillan et al., 2005). It is safe to say that all cellular and organelle membrane contain these five major groups of phospholipids, but considerable variability in proportion exist (Stanley, 1991). Further discussion on membrane phospholipids will be provided in the “SR membrane oxidation leading to accelerated calcium leakage” section.

Other than phospholipids, cholesterol is the other lipid that makes a significant contribution to membrane functionality. Cholesterols are embedded in between the fatty acid tails of membrane phospholipids (Stanley, 1991). Because of its rigid ring structure,

cholesterol can act as a buffering agent that helps the membrane to maintain constant fluidity during temperature fluctuation (Sułkowski et al., 2005). In a membrane that is rigid due to high content of saturated fatty acids or below the transition temperature (the temperature at which membranes switch from fluid to a rigid state), cholesterol increases membrane fluidity by interfering with interactions between fatty acid chains. In fluid membrane, cholesterol stabilizes membrane fluidity by fitting into the gaps created by the kinks in unsaturated fatty acid tails (Presti, 1985).

Other than serving as a barrier, membranes also serve many other vital properties like transport, synthesis, secretion and cell recognition. However, that cannot be achieved by lipids alone; membrane proteins are needed to carry out these specific functions of the plasma membrane (Stanley, 1991). Many types of proteins are found near or attached to the membranes. Yeagle (1987) classified them into integral membrane proteins and peripheral membrane proteins. Integral membrane proteins are those that are anchored or buried in various depths into the lipid bilayers, which can only be released by treatments that physically disrupt the phospholipid bilayer. On the other hand, peripheral membrane proteins are indirectly associated with membranes through protein-protein interactions that can removed by gentle laboratory procedures like washing or altering ionic strength/pH (Cooper, 2000; Yeagle, 1987). Integral membrane proteins are not distinguished from other group of proteins by their structures or functions, but rather by the location of this group. Some of the integral membrane proteins are anchored in the outer leaflet of the plasma membrane, while some are anchored in the inner leaflet, and the others are transmembrane proteins (Von Heijne, 1992). The integral membrane protein functions can range from cell to cell recognition to active transport. Conversely,

peripheral proteins consist mainly of metabolically active proteins, such as glycolytic enzymes and certain cytochromes. Much less is known about membrane protein than lipids because physically disruptive procedures are sometimes required to isolate these proteins (Ostermeier and Michel, 1997).

**Membrane lipid oxidation.** Larick and Turner (1990) and Noci et al. (2005) have demonstrated that PUFA are predominately associated with the phospholipid fraction of the membrane. Consequently, lipid oxidation is expected to occur sooner or later in biological membranes. Pikul et al. (1984) estimated that the phospholipid fraction contributed about 90% of the malonaldehyde measured in total fat from chicken meat. Igene et al. (1980) further showed the PUFA content of phospholipids was positively correlated to the development of rancidity. Finally, Yin and Faustman (1994) concluded that the level of lipid oxidation is more strongly influenced by oxidative stability of membrane components rather than that of sarcoplasmic components.

Membrane lipid oxidation occurs the same way as in other components of the cell. Membrane phospholipids can undergo oxidation through enzymatic or nonenzymatic mechanisms, which both lead to the generation of free radicals and eventually oxidized lipid. Transitional metal ions, heme compounds, and radiation are all accelerating agents in this reaction (Stanley, 1991). The content, composition, and quality of dietary fat in feed and the tendency of animal species to incorporate fatty acids into membrane phospholipids all affect the fatty acid composition of membranes, and their susceptibility to lipid oxidation (Ahn et al., 1995). For example, diets rich in PUFA are known to increase the proportion of PUFA in the phospholipid portion of beef (Dannenberger et al.,

2006), fish (Huang et al., 1998) and pork (Nurnberg et al., 1998), and thus result in more lipid oxidation unless antioxidants are supplemented.

Once oxidation of lipids is initiated and considerable quantities of ROS are propagated, a whole range of biological reactions occur that can have catastrophic consequences for the membrane. The effects of membrane lipid oxidation are seen in both the membrane itself as well as in the surrounding cellular environment (Gutteridge and Halliwell, 1990). O'Brien (1987) listed some representative effects of lipid oxidation on membrane function, which includes increased permeability, decreased fluidity, inactivation of membrane-bound enzymes and cross-linking of membrane proteins. These effects can cause an alteration in organelle functions such as uncoupling of oxidative phosphorylation in mitochondria and increasing calcium leakage from the SR.

**SR membrane oxidation leading to accelerated calcium leakage.** Senaratne (2012) reported that meat from cattle fed a high WDGS diet has higher free calcium levels in the sarcoplasm compared to cattle fed a corn-only diet, and this result triggered the materialization of this research. Van Zutphen and Cornwell (1973) used model lipid bilayer membranes to demonstrate that lipid oxidation causes an increase in membrane permeability, which in turn decreases the stability of the membrane. Machlin and Bendich (1987) also have documented that lipid oxidation occurs at an accelerated rate in cell membranes containing more PUFA, causing the membrane structure to collapse. It is likely that the increase of PUFA from WDGS contributes to oxidation of SR membrane and causes the difference in membrane permeability that leads to the difference in sarcoplasmic free calcium concentration between the two treatments.

Many studies have found a shift of lipid profile from phospholipid to neutral lipid in plasma membranes when rats were changed from a saturated fat diet to a high unsaturated fat diet (Brasitus et al., 1985; Deaver Jr et al., 1986; Thi-Dinh et al., 1990). Ji and Takahashi (2006) found that sarcoplasmic free calcium content increases with decreasing phospholipid content of SR membrane during aging of pork and beef. They hypothesized that degradation of phospholipids occurred during aging, allowing stored calcium to leak into the sarcoplasm through channels formed by the liberation of phospholipids. It has been suggested that phospholipases increase activity to remove esterified fatty acids when lipid oxidation occurs, thus resulting in phospholipid liberation (Leshem, 1987; Mead et al., 1980).

Cheah and Cheah (1985) used pork from pigs with malignant hyperthermia as a model to further comprehend how the initial increase of free calcium can trigger the release of more calcium into the sarcoplasm. Specifically, the initially-released free calcium activates calcium-dependent phospholipase A2 for the hydrolysis of phospholipid to alter the membrane permeability of SR, causing further release of calcium into the sarcoplasm. This cycle of "calcium induced calcium release" will continue on from early postmortem until the depletion of calcium sources. This is different from the traditionally known "calcium induced calcium release", which is caused by a change of membrane potential. Instead, Cheah and Cheah (1985)'s model explored the role of endogenous enzymes on the increased level of sarcoplasmic calcium postmortem. Gutteridge and Halliwell (1990) suggested that the increased sarcoplasmic calcium level postmortem can be a more injurious event than direct oxidation of membrane lipid due to calcium's ability to initiate phospholipase A2.

Products of phospholipid hydrolysis include free fatty acids and lysophospholipids (Kagan, 1989). Free fatty acids are known to promote collapse of the mitochondrial electrochemical proton gradient by opening the permeability transition pore (PTP) (Broekemeier and Pfeiffer, 1995; Di Paola and Lorusso, 2006). The PTP is a large, non-selective channel on the membrane that usually only opens under specific conditions (Hunter et al., 1976); as a result, the opening of PTP can further allow free calcium leaking out of the SR. Lysophospholipids are also capable of increasing membrane permeability through stabilizing dimer formation between two peptide helices to form open channels (Lundbaek and Andersen, 1994). Flemming et al. (2006) showed that lysophospholipids activated transient receptor potential cation channels (permeable to calcium, sodium and potassium ions) by supporting the channel's structure. Also, at high levels in plasma membrane, lysophospholipids can lead to cell fusion and even lysis (Weltzien, 1979).

A diet shift can also result in a shift of phospholipid profile. Dannenberger et al. (2006) showed that a pasture diet high in PUFA significantly increased lysophosphatidylcholine in the muscle tissue of German Simmental bulls compared to the muscle tissue from animals fed a concentrate diet. It is possible that feeding diets high in PUFA incorporated more PUFA in the phosphatidylcholine (PC), thus increase the oxidation rate of PC, leading to an increased proportion of lysophosphatidylcholine. Moreover, Mlekusch et al. (1993) found a decrease in phosphatidylethanolamine (PE) and an increase in PC in rat liver when rats were fed a diet high in PUFA (corn oil), while Beare and Kates (1964) found that rat muscle PC can incorporate linoleic acids better than PE can. Mello et al. (2012) showed that feeding WDGS increased linoleic acid

content of beef muscle tissue by 53%. Shifts in phospholipid profile found by Mlekusch et al. (1993) can be explained by the increase of linoleic acid content. Phospholipid PE has the ability to enhance the activity of the SERCA in SR membranes through specific headgroup interactions with the structure of SERCA (Hunter et al., 1999). Therefore, it is also possible the increase in sarcoplasmic free calcium concentration found in Senaratne's (2012) study is the direct result of a SR phospholipid profile shift, where the lack of PE in WDGS-fed cattle impeded the rates of calcium influx pre-rigor.

**Role of dietary antioxidants in our hypothesis**

Antioxidants delay the onset and/or reduce the rate of oxidation. Antioxidants work either by inhibiting the formation of free radicals in the initiation step or interrupting propagation of the free radical chain. Most dietary antioxidants are phenolics or polyphenolics, and phenolics can act as hydrogen donors in the reaction because the phenol groups form radical intermediates with delocalized electrons (Pereira et al., 2009). When the unsaturated fatty acids undergo oxidation, an antioxidant can block this oxidation by supplying a hydrogen in the first free radical formed, thereby reconverting it to the original fatty acid (Hayes et al., 2010; Mancini and Hunt, 2005; Nam et al., 2006).

Antioxidants can easily be incorporated into animal tissues through dietary means. Therefore, feeding antioxidants along with WDGS is a great way to generate samples with varying degrees of membrane lipid oxidation capacity. In our hypothesis, feeding antioxidants adds stability to the SR membrane, slowing the oxidation of membrane lipid. Although the actual mechanism of oxidation-influenced membrane degradation is unclear, Senaratne (2012) showed increased release of calcium post-rigor

and delayed and/or reduced proteolysis in beef from antioxidant-supplemented steers.

Dietary antioxidants, natural or synthetic, are widely recognized for their ability to increase post-rigor oxidative stability in meat (Santé-Lhoutellier et al., 2008; Ventanas et al., 2006). There are currently two main types of antioxidant supplements in ruminant diets: natural antioxidants like vitamin E and synthetic antioxidant blends like Agrado-Plus (Novus International, Saint Charles, MO).

**Types of antioxidants.** Vitamin E is an essential nutrient that functions as an antioxidant in animals and is found in plants and seeds. Eight molecules, four tocopherols and four tocotrienols, have antioxidant activity. Out of the eight, α− tocopherol has the most antioxidant activity in biological systems (Liu et al., 1995). In general, vitamin E is commonly supplemented to the diets of finishing cattle as α−tocopheryl acetate. Although the acetate ester lacks antioxidant activity, the acetate component is cleaved by intestinal esterases, leaving α-tocopherol which is promptly absorbed across the intestinal wall into the mesenteric lymphatic system. From there, α-tocopherol is transported by lipoproteins to muscle cells, where it is then deposited in the membrane (Schaefer, 2007). The chromanol ring of α−tocopherol is located within the polar head group of phospholipids and the phytol side chain interacts with the unsaturated fatty acid chain toward the interior of the membrane (Fukuzawa and Fujii, 1992). Although α-tocopherol is presented in all cellular membranes, it is interesting to note that beef mitochondrial and SR membrane contain 4.5 and 6 fold greater α−tocopherol concentration, respectively, compared to intact muscle. However, when cattle (Arnold et al., 1993) and hogs (Asghar et al., 1991) were supplemented with α-tocopherol, proportionally less of the

supplemented α-tocopherol was deposited into the mitochondrial (8%) and SR (7%) membrane in comparison to intact muscle (70%) tissue. This suggests that mitochondrial and SR membranes are the preferred site for α-tocopherol disposition, but because of that, mitochondrial and SR membranes reach their thresholds for α-tocopherol sooner than muscle tissue when the animals are supplemented with α-tocopherol.

Alpha-tocopherol can maintain cellular integrity and delay lipid and pigment oxidation effectively (decreased off-flavor and metmyoglobin formation) when used as a supplement in cattle (Maddock et al., 2003; Senaratne et al., 2011), pigs (Hoving-Bolink et al., 1998; Monahan et al., 1992; Pfalzgraf et al., 1995), and lambs (Santé-Lhoutellier et al., 2008). However, α−tocopherol accumulation in muscle is influenced in a dose and duration manner. It is suggested that when producing beef for domestic markets, 500 IU vitamin E/hd/d should be supplemented for the last 100 d prior to harvest to achieve the desired tissue levels. For export markets, the common goal is to provide 1,000 IU vitamin E/hd/d for the same 100-d finishing period (Schaefer, 2007). Compared to most antioxidants in the market for ruminant supplementation, vitamin E is the most studied and established product to improve marketability of fresh beef products.

Agrado-Plus (AG) is a beef cattle antioxidant supplement manufactured by Novus International (Novus International, Saint Charles, MO) which contains a very strong antioxidant mixture of ethoxyquin (1,2-dihydro-6ethoxy-2,2,4-trimethylquinoline) and TBHQ (tertiary butylhydroquinone). Ethoxyquin is an effective fat-soluble phenolic antioxidant found in feeds rich in carotenoids, and is widely used as a preservative in animal feed because of its high antioxidant efficiency and stability as well as low cost of synthesis (Blaszczyk et al., 2013). In the cattle industry, ethoxyquin is approved for use

to reduce oxidation in high-fat feed ingredients in feedlot operations (Maddock et al., 2003). One other key function of ethoxyquin is to stabilize fat-soluble vitamins such as vitamin E, which creates additive antioxidant effects when ethoxyquin is fed in combination with vitamin E (Lauridsen et al., 1995). Although most ethoxyquin is broken down in the animal body and excreted in the urine, small amounts of ethoxyquin can be detected in liver, kidney, adipose and muscle tissue (Burka et al., 1996). Therefore, ethoxyquin may still play a role as an antioxidant in fresh meat products. On the other hand, TBHQ is a water-soluble, phenolic antioxidant usually added in conjunction with butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate (PG) to provide a synergistic antioxidant effect in feeds high in unsaturated fat (FDA, 1999). Due to its water soluble nature, TBHQ disappears from the plasma pool rapidly (Domingos et al., 2007). Therefore, dietary TBHQ likely has minimal effects on fresh meat quality.

The antioxidant effect of AG is still up for debate. Krumsiek and Owens (1998) reported that feeding AG short term to cattle increased the lipid and color stability of ground beef and ribeye steaks during retail display while Walenciak et al. (1999) found no significant improvement in color stability in top loin steaks and ground beef during retail display from feeding AG long-term. In a thorough study of feeding AG to cattle, Senaratne (2012) reported a small, but significant decrease in beef tenderness. Little information is available regarding the effects or mechanisms of the antioxidant blend of ethoxyquin and TBHQ on membrane stability.

**Dietary antioxidants as an approach to prevent membrane lipid oxidation.** Different

antioxidants provide different mechanisms of scavenging and reducing free radicals. Alpha-tocopherol incorporated into cell membranes directly intercept free radicals where the oxidation process starts. Because of the advantageous subcellular location of α-tocopherol, it functions very efficiently to protect the unsaturated fatty acids in phospholipids from oxidation (Liu et al., 1995; Palozza and Krinsky, 1992). Alpha-tocopherol reduces a neighboring free radical by donating an electron from the hydroxyl group on carbon 6 of the chromanol ring (Schaefer et al., 1995). As a consequence of oxidation, there is formation of two new molecules, α-tocopheroxyl and α-tocopherol quinones. These molecules have no oxidative properties; therefore, oxidation of lipid is not allowed to continue (Botsoglou et al., 2003). In addition, Schaefer et al. (1995) showed that integration of α-tocopherol into the membranes can prevent oxidized free fatty acid migration into the sarcoplasm. Alpha-tocopherol is not only effective in preventing membrane lipid oxidation, but also helps contain oxidized lipid from interacting with other intact lipid and proteins within the cell. Finally, Cheah et al. (1995) suggested α-tocopherol's ability to stabilize membranes is associated with inhibition of phospholipase A2 activity. As described earlier, phospholipase A2 can be activated by sarcoplasmic calcium; alpha-tocopherol's ability to inhibit phospholipase A2 assists in maintaining membrane integrity (Cheah et al., 1995).

Unsaturated fatty acids in mitochondria and microsomal (mainly SR) membranes are thought to be the origin of lipid oxidation in muscle (Monahan et al., 1990; Monahan et al., 1994), so it makes sense that the higher concentrations of α-tocopherol found in these two regions suppresses the initiation of oxidation (Arnold et al., 1993). As discussed in the previous section, the mitochondrial and SR membranes reach their

threshold level for α-tocopherol sooner than muscle tissue because they are the preferred α-tocopherol deposition sites. Although only minimal amounts of supplemental α-tocopherol are known to incorporate into the SR membrane, Cheah et al. (1995) showed that dietary supplementation of α-tocopherol reduced free calcium release in pork. It is possible that different animals from different trials may have access to different amount of natural sources of vitamin E, which would explain the variation in benefits from supplemental α-tocopherol.

Feeding animals oxidized feed ingredients can result in reduced animal weight gain, poor feed conversion, increased disease susceptibility, and greater mortality (Miller et al., 1993). Therefore, synthetic antioxidants such as ethoxyquin and TBHQ are widely used for protection of the feed ingredients against oxidative degradation during storage and processing (McCarthy et al., 2001; Tavárez et al., 2011). Similar to α-tocopherol, ethoxyquin and TBHQ neutralize free radicals through donation of one or two electrons. During oxidation, ethoxyquin forms 2,6-dihydro-2,2,4-trimethyl-6-quinolone and 1,8′-di *(*1,2-dihydro-6-ethoxy-2,2,4-trimethyl-quinoline) (He and Ackman, 2000), and these oxidation products still possess antioxidant activity (de Koning, 2002). Major metabolites of TBHQ are TBHQ−glucuronide and TBHQ−sulfate as well as a trace of unchanged TBHQ (Peters et al., 1996). It is important to note that both ethoxyquin and TBHQ work best at low concentrations (<150 mg/kg and <200 mg/kg in feed, respectively). In low concentration, these metabolites are rapidly excreted and show no adverse effect on human or animal health. However, formation of free radicals can occur when ethoxyquin and TBHQ are administered in high concentrations; adverse effects were observed when fed over 100 times of the recommended dosages to rats, mice and

dogs (Drewhurst, 1998). Other factors like presence of transition metals and change in solubility and pH can also convert these antioxidants to phenxyl radicals and contribute short- or long-term toxicities to animals or people consuming meat from these animals (Sakihama et al., 2002; Skaare and Henriksen, 1975).

Although both ethoxyquin and TBHQ showed abilities to reduce ROS in animal feed, minimal amount of ethoxyquin and TBHQ are preserved in animal tissues (Burka et al., 1996; WHO, 1975). Senaratne et al. (2011) further showed that AG itself was not sufficient to overcome oxidation from elevated PUFA in muscle. Synthetic antioxidants like AG likely have minimal effects on fresh meat quality when fed without an elevated supply of vitamin E. Both ethoxyquin and TBHQ are known for their ability to retard oxidation of carotene, xanthophyll and vitamins (Blaszczyk et al., 2013; Van Esch, 1986). When fed in combination with α-tocopherol, ethoxyquin has demonstrated a sparing effect on α-tocopherol level in animal tissue (Lauridsen et al., 1995). Chickens fed ethoxyquin have significantly higher α-tocopherol content in the plasma compared to chickens fed a feed not stabilized with ethoxyquin (Bartov and Bornstein, 1981). On top of preserving α-tocopherol stability in feed, Lauridsen et al. (1994) further suggested that the small amount of ethoxyquin preserved in animal tissues might continue to protect α-tocopherol from oxidative deterioration. However, Maddock et al. (2003) concluded that there are no synergistic effect between ethoxyquin and α-tocopherol based on a study of retail display color of beef. On the other hand, TBHQ is rapidly absorbed and progressively excreted in the urine (Madhavi and Salunkhe, 1995). Unless administered directly to meat products (Chastain et al., 1982), it is highly unlikely TBHQ has any metabolic effect in preventing membrane oxidation.

**Myofibrillar protein degradation**

It has been known for a long time that meat tenderness improves over time during postmortem storage. However, the mechanism has not been fully described until recently. Enzymatic (Boehm et al., 1998; Koohmaraie, 1989) and non-enzymatic calcium (Takahashi, 1992; Takahashi, 1996) theories have been proposed and extensively studied. The non-enzymatic calcium theory proposed that the rise in free calcium in postmortem muscle weakens Z-disks, the linkage between actin and myosin, and disconnects several structural proteins like titin, nebulin, and desmin from the sarcomere (Ji and Takahashi, 2006; Takahashi, 1996). However, this non-enzymatic calcium theory is less likely to occur than the enzymatic theory of postmortem meat tenderization. In fact, it is now well established that the enzymatic theory (fragmentation of myofibrillar and cytoskeletal proteins by proteases) is responsible for the tenderization of meat, but it is a matter of debate of which proteins are degraded and which enzymes are responsible for the process (Koohmaraie and Geesink, 2006; Ouali et al., 2006). Since this dissertation research also aimed to understand how the release of free calcium affects the mechanism of beef tenderization, understanding the many factors influencing postmortem meat tenderization is a must.

**Muscle structure.** Muscle cells or myofibers are highly organized because they are required to perform a diverse array of mechanical functions, from movement of limbs to maintaining balance and coordination. They are also the only cells that are required to generate this much force and movement (Huijing, 1999). In order to understand

postmortem muscle protein degradation, we must first understand muscle cell structure. Muscle cells are composed of small contractile units known as myofibrils. The myofibril is a string-like structure made of repeating units called sarcomeres. These sarcomeres are the most basic units of muscle cells, and sarcomeres are made of structural and functional proteins needed to perform the contraction and relaxation at the molecular level (Du and McCormick, 2009).

Myofibrils appear striated under a microscope. This is because myofibrils are made up by protein dense A-bands and less-protein-dense I-bands. A dark line bisecting each I-band is known as a Z-disk, and in the center of the A-band is the M-line. A sarcomere is the area between two Z-disks, which compose of one complete A-band and two halves of I-bands. The I-band is made up primarily of thin filaments while the A-band is made up of thick filaments and some overlapping of thin filaments (Du and McCormick, 2009).

There are more than 65 proteins that make up the structure of a sarcomere (Fraterman et al., 2007). Most of the proteins interact with one another in a highly coordinated fashion. The sarcomere is comprised of the thin (mostly actin) filaments, the thick (mostly myosin) filaments, and the giant filamentous molecule titin. The thin filaments are anchored in the Z-line, where they are cross-linked by α-actinin. The thick filaments are centrally located in the sarcomere and constitute the A-band. The myosin heads, or cross-bridges, on the thick filament interact with actin during contraction (Du and McCormick, 2009). Titin spans from the Z-line to the M-line, thus forming a third sarcomeric filament. In the I-band region, titin is extensible and functions as a molecular spring that develops passive tension upon stretch. In the A-band, titin is inextensible due

to its strong interaction with the thick filament (Fritz and Greaser, 1991).

Troponin and tropomyosin are key proteins on the thin filament that control contraction (Farah and Reinach, 1995). The presence of calcium facilitates a conformational change of troponin and tropomyosin. When troponin C is bound to calcium, it pulls troponin I away from actin, shifting tropomyosin from blocked-state to closed-state, allowing the interaction of myosin and actin. During contraction, actin and myosin form a tight bond, known as the actomyosin bond, in order to pull the thin filaments closer to the M-line (Du and McCormick, 2009; Farah and Reinach, 1995). In the presence of ATP, calcium is actively sequestered back into the SR and the contractile process is halted. As calcium concentration drops, calcium is released from troponin C, and tropomyosin is shifted back to the blocked state. This again blocks the myosin binding site of actin, causing the muscle to relax. In post-rigor muscle, the actomyosin bond becomes permanent because there is no more ATP available to release the bond (Aberle et al., 2001; Du and McCormick, 2009). This is a very general description of sarcomere structure; the complete microstructure of a sarcomere is too complex to be described in this section. More descriptions of myofibrillar proteins and their function will be discussed in "the mechanism of tenderization" section.

**Proteinase system.** During aging, key cytoskeletal and regulatory proteins such as troponin I, troponin T, desmin, vinculin, meta-vinculin, dystrophin, nebulin and titin are degraded (Huff-Lonergan et al., 1996; Hwan and Bandman, 1989; Koohmaraie and Geesink, 2006). Postmortem proteolysis of myofibrillar and structural proteins begins soon after the animal is harvested. As the animal carcass chills during rigor development,

early postmortem release of calcium could be expected to enhance tenderness as proteolysis is initiated at warmer temperatures and higher pH (Dransfield, 1994; Geesink et al., 2000). Although the proteases exhibit proteolytic activity during extended postmortem storage, the bulk of their effect on meat tenderness is observed within the first week postmortem (Eilers et al., 1996; Purchas et al., 1999; Smith et al., 1978).

The calpain system, cathepsins, the muticatalytic proteinase complex, and caspases have all been studied in relation to postmortem protein degradation (Kitamura et al., 2010; Koohmaraie, 1994; Taylor and Koohmaraie, 1998; Underwood et al., 2008). Uytterhaegen et al. (1994) showed that when muscle was injected with calpain inhibitors E64 and leupeptin, myofibrillar protein degradation was reduced with no reduction in shear force over time. On the other hand, injection of cathepsin inhibitor - peptidyl-diazomethane - in meat showed no effect. The meat still exhibited protein degradation and reduced in shear force (Hopkins and Thompson, 2001a). One interesting note is that partial troponin T degradation even with no shear force reduction was still observed in the presence of calpain inhibitor E64 (Hopkins and Thompson, 2001b; Uytterhaegen et al., 1994). This could indicate that other proteases may also play a minor role in protein degradation, if not tenderization. Koohmaraie and Geesink (2006) concluded the calpain system is the only major contributor to meat tenderization.

The calpain system is composed of two major isoforms: μ-calpain and m-calpain. Both μ and m-calpain are heterodimers composed of an 80 kD and a 28 kD subunit (Suzuki, 1990). The function of the 28 kD subunit is unknown, while the 80 kD subunit contains four domains and each domain has a specific function (ranging from catalytic activity to calcium binding site), which suggest that the conformational states of μ and m-

calpain may have a direct effect on their activity (Hosfield et al., 1999; Strobl et al., 2000). One domain of particular interest is domain 3; it is a calcium binding domain that regulates the activity of calpains by participating in critical electrostatic interactions and the binding of phospholipids phosphatidylinositol (Tompa et al., 2001). Perhaps the liberation of membrane phospholipid during oxidation also stimulates the activity of calpains.

The functionality of the calpain system in postmortem muscle is influenced by several factors, mainly pH, sarcoplasmic calcium level, temperature and inhibitors (calpastatin) (Huff Lonergan et al., 2010). *In vitro* work suggests that calpain activity is maximal at 25 $^{o}$C with a pH of 7.5 (Du and McCormick, 2009). However, this is not present in postmortem conditions. Gradual postmortem pH decline of muscles favors the activation of μ-calpains (Carlin et al., 2006; Melody et al., 2004), while rapid pH decline negatively affects calpain activity as low pH tends to denature the calpains (Barbut, 1996; Bee et al., 2007). Boehm et al. (1998) suggested only 10% of calpain activity is left after 24 hours postmortem at 5$^{o}$C with a pH of 5.5. However, Koohmaraie et al. (1986) showed calpains retained 24-28% of their maximum activity at pH 5.5 to 5.8 and 5$^{o}$C, and activity could still be detected at 56 d postmortem. (Both studies compared to the maximum activity at 25 $^{o}$C with a pH of 7.5). In addition, Lee et al. (2000) detected higher calpain activity in *Biceps femoris* and *Semimembranosus* muscles injected with sodium pyrophosphates plus sodium chlorides as sodium pyrophosphates and salt slowed down pH drop of postmortem muscles due to their buffering actions.

In general, the calpain system requires the presence of calcium to be activated. Mu-Calpain requires between 5 to 65 μM of calcium for half-maximal activity, and m-

calpain requires between 300 to 1,000 μM of calcium for half-maximal activity (Goll et al., 1992). Ji and Takahashi (2006) reported that in rabbit *psoas major* muscle, free calcium concentration increased from 33 μM to 230 μM at 30 hours of postmortem, and in beef and pork muscles, free calcium increased from 16 μM (at 40 min postmortem) to 210 μM (at 3 d postmortem). Parrish et al. (1981) reported that free calcium in beef longissimus muscle at 10 - 14 d aging was 638 - 970 μM. Finally, Senaratne (2012) documented that free calcium increased from 791 to 947 μM from 8 d aging to 28 d aging. Calcium has a major role in the tenderization of meat, and it is in general considered that the higher the free calcium concentration, the more calpain activity occurs.

A third isoform of calpain, calpain 3, (also known as P94) was discovered in the 1990's. Calpain 3 is a 94 kD protein whose structure is homologous to μ and m-calpain, and shares the similar properties of calcium-dependent activation and maximal activity at neutral pH (Goll et al., 2003). However, calpain 3 is very unstable and undergoes fast autolysis; therefore, the functionality of this third isoform in postmortem muscle has not been demonstrated (Kinbara et al., 1998; Sorimachi et al., 1989).

Finally, calpastatin is an endogenous inhibitor of the calpain system. Interestingly, it also requires calcium to bind to the calpains (Huff-Lonergan et al., 2010). In general, less calcium concentration is required for calpastatin to bind to calpains (40 μM for μ-calpain and 250 to 500 μM for m-calpain) than the calcium concentration required for calpains to reach half-maximal activity, suggesting that if calpains and calpastatin were both presented in sarcoplasm, rising calcium concentrations would result in binding of calpastatin to calpains before it could initiate proteolytic activity (Kapprell and Goll,

1989). This result fits perfectly with the Melloni et al. (2006) concept that in order to prevent a large proportion of inactive calpains to undergo activation following a rapid rise in sarcoplasmic calcium, sarcoplasmic calpastatin binds to the still inactive calpains. Thus, the sarcoplasmic calpastatin pool plays a fundamental role in the control of the number of calpain enzymes susceptible to activation. Calpastatin autolyzes over time and allows the inactive calpains to function as free calcium concentration continues to rise (Miller et al., 2011). Therefore, with time, increases in myofibrillar protein degradation and reductions in shear force are still observed (Wendt et al., 2004). Calpastatin has a molecular mass of 60 - 70 kD, and it contains four inhibitory domains, each of which can block the active site of both μ and m-calpains (Goll et al., 2003; Kemp et al., 2010). Furthermore, calpastatin is degraded by calpains in postmortem muscle. Specifically, calpains cleave the weaker inhibitory domains of calpastatin, creating specific peptide fragments that retain inhibitory activity (Doumit and Koohmaraie, 1999; Mellgren, 2008). It has now been confirmed that a high level of calpastatin is associated with the decrease in postmortem proteolysis, which leads to tougher meat (Kent et al., 2004). Therefore, calpastatin is now widely used as a tenderness biomarker (Zór et al., 2009).

**Mechanism of tenderization.** Research showed that calpains degrade many structural and functional myofibrillar and cytoskeletal proteins during postmortem aging, but have minimal effects on contractile proteins such as actin or myosin (Dayton et al., 1976; Goll et al., 1991). Early research work suggested that Z-disk degradation is responsible for the postmortem tenderization (Davey and Gilbert, 1968). However, Taylor et al. (1995) showed that it is not the Z-disk that is degraded, but the degradation of proteins near the

Z-disk. Several ultrastructural studies showed that muscle fibers break in the I-band region near the Z-disk, but the Z-disk itself is stable (Abbott et al., 1977; Davey and Graafhuis, 1976).

The largest protein in muscle fiber, titin, is crucial to the structure of sarcomere. A single titin extends from the Z-disk all the way to the M-line (Du and McCormick, 2009). Nebulin, another mega-protein, is part of the thin filament and has been hypothesized to aid the anchoring of thin filaments to the Z-disk (Robson et al., 1997). Research has shown that both titin and nebulin can be degraded during postmortem storage, which likely weakens the sarcomere structure at the I-band, giving the degradation of titin and nebulin direct linkages to the increase of tenderness (Wheeler and Koohmaraie, 1994).

The costamere is an important structural component of the muscle cell; it connects Z-disks to the sarcolemma (Du and McCormick, 2009). Taylor et al. (1995) demonstrated using electron microscopy that the sarcolemma can be detached from the muscle fiber, and such detachment is likely due to the degradation of the costamere. The costamere is made up of structural proteins like vinculin, talin, desmin, dytrophin and β-spectrin (Du and McCormick, 2009). Many research studies have shown vinculin and desmin are readily degraded during postmortem storage, and their degradations also have a direct relationship to increase in tenderness and myofibrillar fragmentation index (Taylor et al., 1995; Taylor and Koohmaraie, 1998). Filamin, another very large, but less studied protein, is also important to the muscle cell structure. Although filamin only accounts for 0.1% of total muscle protein, filamin is located at the surface of the Z-disk. Degradation of filamin can disrupt linkages connecting the muscle fibers to the sarcolemma, having a similar effect as the degradation of the costamere, assisting the detachment of

sarcolemma to muscle fibers (Robson et al., 1997).

Troponin T has also been shown to degrade during postmortem storage (Huff-Lonergan et al., 1996). However, troponin T itself is not a structural protein, and it likely contributes nothing to postmortem tenderization. Therefore, the degradation of troponin T is nothing but an excellent indicator for postmortem protein degradation (Kemp et al., 2010; Kitamura et al., 2010). Finally, actin and myosin are known to be resistant to μ-calpain degradation. However, Lametsch et al. (2004) used an extremely sensitive evaluation method (2D gel electrophoresis) and peptide-mass spectrophotometer mapping to demonstrate that minor changes in the myosin heavy chain and actin occur during degradation. Since myosin and actin are the primary proteins in muscle structure, even small changes on the actomyosin bonds may have potentially great effect on meat quality.

**Conclusion**

This literature review provides evidence for a significant role for membrane integrity on the tenderization mechanism in beef from cattle fed a high concentration of WDGS. The complexity of this subject, thus raises a number of questions that need to be answered to ascertain the role for membrane integrity. Does feeding a diet high in PUFA alter PUFA content of the SR membrane? If so, what are the relationships between SR membrane composition, oxidation, proteolysis, and beef tenderization? If, as hypothesized, a high PUFA diet destabilizes the SR membranes and influences timing and/or extent of calcium release, and the tenderization process is affected by free calcium concentrations, would feeding an antioxidant lead to a different result? Finally, how do we utilize the findings to optimize beef quality?

Animal tissues react quickly from lack of oxygen, and the accumulation of waste products occurs soon after death. High concentrations of unsaturated fatty acids in SR and mitochondrial phospholipids could cause the membranes to quickly lose their integrity, leading to the early postmortem release of previously-sequestered calcium. Membrane lipid and protein oxidation can result in many pathways of increased calcium release. They are summarized as follows:

1) Oxidized RyR1 becomes active at low luminal calcium concentrations and is not inhibited by high sarcoplasmic calcium concentration.

2) Oxidation leads to increased hydrolysis of phospholipid phosphatidylinositol, which increases the production of IP3 to activate the IP3 receptor channel.

3) A diet high in PUFA shifts membrane phospholipid profile by increasing PC and decreasing PE. Phospholipid PE can enhance catalytic activity of SERCA, while SERCA activity can be hindered by the lack of PE.

4) Oxidation of the unsaturated fatty acids in phospholipids.

5) Oxidation leads to an increase in phospholipase activity to further degrade phospholipids.

6) Free fatty acids and lysophospholipids from phospholipid degradation can also increase membrane permeability through different mechanisms.

Dietary α-tocopherol has proven to improve oxidative stability of fresh meat products, while the effects of synthetic dietary antioxidants like ethoxyquin and TBHQ on fresh meat quality are still debatable. Membrane-bound α-tocopherol has been shown to quench ROS to restore oxidized fatty acids to their original state, thus preserving membrane integrity, while some research further showed membrane-bound α-tocopherol

can inhibit phospholipase activity. On the other hand, ethoxyquin and TBHQ mainly work through stabilization of feed ingredients to preserve the integrity of α-tocopherol by creating an E-sparing effect. Further research is needed to fully understand the role of antioxidants on redox activities at the membrane-sarcoplasm interface.

There is potential for long-range improvement in U.S. agriculture and food systems from this research. Lipid oxidation and discoloration are likely indicators of differences in membrane stability. If they are indicators, it might be possible to manipulate these conditions to enhance tenderness without compromising meat quality. The results could lead to new carcass management strategies that enhance beef tenderness while maintaining quality. It is clear that animal nutrition has an important role in fresh meat quality beyond just gross composition of the carcass.

## MATERIALS AND METHODS

### Manuscript 1 - Feeding wet distillers grains plus solubles contributes to sarcoplasmic reticulum membrane instability

**Animals.** A total of 96 steers were used in a randomized complete block design with two treatments. Steers were fed at University of Nebraska feedlot (Mead, NE) for 147 d on either a corn-only diet or a corn-based diet with 50% WDGS (DM basis; Appendix I). Steers were also implanted on d 1 with Revelor-XS (Merck Animal Health, Summit, NJ). Animals were blocked by BW, stratified by BW within each block, and assigned randomly to pens. Pens were randomly assigned to one of the two treatments with six pens per treatment and eight steers per pen.

**Sample collection and preparation.** All steers were harvested at a commercial abattoir (Greater Omaha Packing Co., Omaha, NE) on October 27, 2012. After 48 h of postmortem chilling, 30 out of 96 carcasses were selected with 2 to 3 carcasses from each pen (15 carcasses from each treatment). Carcass selection was based on treatment and quality grade (low Choice). Strip loins (*Longissimus lumborum*) from all selected carcasses were collected, vacuumed-packaged and transported to Loeffel meat laboratory at the University of Nebraska-Lincoln. Strip loins from the left sides of the carcasses were fabricated on postmortem aging d 2 and 7, and strip loins from the right sides of carcasses were fabricated on postmortem aging d 14 and 21. Each strip loin was fabricated into 2 tenderness samples (2.54 cm) and 3 lab samples (1.27 cm) for each aging period, from the anterior to the posterior end of the loin muscle. The remainders of

the strip loins were immediately vacuum packaged in vacuum pouches (3mil STD barrier, Prime Sources, St. Louis, MO) on a Multivac Packaging machine (Mutivac C500, Multivac, Kansas city, MO) and aged to the next designated aging period. Upon fabrication, steaks subjected to retail display conditions (2 ± 2$^{o}$C, and exposed to continuous 1,000-1,800 lux warm white fluorescence lighting) were packaged in Styrofoam trays (Styro-Tech, Denver, CO) and overwrapped with oxygen permeable polyvinyl chloride film (PSM18, Prime Source, St. Louis, MO). Samples for tenderness assessments, free calcium concentrations and proteolysis were obtained on d 0 and 7 of retail display for each aging period, and samples for oxidation assessments were obtained on d 0, 4 and 7 of retail display for each aging period. Sarcomere length was determined on samples with 0 d retail display after 2 d of aging. For SR membrane fatty acid, phospholipid, neutral lipid and total lipid analyses, samples were obtained at d 0 of retail display after 14 d of aging. Samples were vacuum packaged and frozen at -20$^{o}$C (tenderness samples) or -80$^{o}$C (lab samples) until analysis. The lab samples were pulverized in liquid nitrogen with a blender (model 51BL32, Waring Commercial, Torring, CT).

**Warner-Bratzler Shear Force (WBSF).** Steaks were removed from the freezer and thawed at 4°C for 24 h prior to grilling. An insulated type T thermocouple (5SC-TT-T-30-120, OMEGA Engineering, Inc., Stamford, CT) was inserted into the geometric center of each steak and attached to an OMEGA 450-ATT thermometer (OMEGA Engineering, Inc., Stamford, CT) to monitor the internal temperature of the steak. Steaks were grilled on a Hamilton Beach Indoor-Outdoor grill (Model 31605A, Proctor-Silex, Inc.,

Washington, NC), flipped once when the internal temperature reached 35°C, and removed from the grill when they reached an internal temperature of 71°C. Grilled steaks were cooled at 4°C for 24 h, and six cores, 1.27 cm in diameter, were removed parallel to the muscle fibers using a drill press. Cores were sheared on a Texture Analyzer (model TMS-PRO, Food Technology Crop., Sterling, VA) with a Warner-Bratzler blade. The mean peak shear force (kg) of 6 cores was calculated for each steak.

**Free calcium concentration.** Free calcium was quantified using the procedure described by Parrish et al. (1981) with modifications. Three g of powdered sample was a transferred to a thick-wall, polyallomer ultracentrifuge tube (13 × 55 mm; Beckman Coluter, Brea, CA) and centrifuged at 196,000 x *g* (Beckman L7-65 Ultracentrifuge with a SW55Ti rotor; Beckman Coluter) at $4^{o}$ C for 30 min. Seven hundred microliters of the supernatant was collected and transferred to an 2 mL Eppendorf tube, treated with 0.1 mL of 27.5% trichloroacetic acid (TCA) and vortexed until the TCA was thoroughly distributed throughout the solution. Samples were centrifuged at 6,000 x *g* (Eppendorf model 5430; Eppendorf, Hamburg, Germany) for 10 min. Four hundred microliters of supernatant were transferred to a syringe, and the volume was brought to 4 mL with deionized distilled water ($ddH_2O$). The diluted calcium sample was filtered through 13 mm diameter Millex-LG 0.20 μm syringe filters (Millipore, Bedford, MA). Calcium concentration of samples was quantified at Ward Laboratories (Kearney, NE) using an inductively-coupled plasma emission spectrometer (iCAP 6500 Radial; Thermo Electron, Cambridge, UK) with appropriate calcium concentration standards.

**Lipid oxidation.** Lipid oxidation was measured by the thiobarbituric acid assay (TBA) described by Ahn et al. (1998) with modifications. Fourteen milliliters of dd$H_2O$ and 1 mL of 10% butylated hydroxyanisole (BHA) in 90% ethanol were added to 5 g of powdered sample. After homogenizing for 15 s using a Polytron homogenizer (model CH-6010; Kinematica, Luzern, Switzerland), the homogenate was centrifuged for 3,000 × *g* for 5 min. One milliliter of homogenate was added to 2 mL of 2-thiobarbituric acid (TBA) and trichloroacetic acid (TCA) mixture (15% w/v TCA and 20 mM TBA in ddH2O) and vortexed for 5 s. The sample mixture was incubated at 70°C in a water bath for 30 min to develop color. The samples was cooled in a cold-water bath for 10 min and centrifuged at 3,000 × g for 15 min. Duplicate 200 μL of supernatant aliquots from each sample were transferred to 96-well microplates (Microtest III sterile 96-well flat-bottomed microplate; Becton Dickinson & Company, Lincoln Park, NJ) and read with a microplate spectrophotometer (Model Epoch, Biotek, Winooski, VT) at 540 nm. All 96-well microplates contained standards to calculate standard curves, and each sample was calculated as mg of malonaldehyde per kg of tissue using the standard curve from each plate.

**Sarcomere length (Appendix II).** Sarcomere length was determined using the powdered sample Helium-Neon laser method described by Cross et al. (1981) with modofications from Dolazza and Lorenzen (2014). A few specks of powdered samples were transferred to a microscope slide. A drop of 0.25M sucrose was applied to the glass slide, and a coverslip was used to cover the samples. The distance between the top of the slide to the base of laser stand was set at 100 mm. A piece of white paper was placed underneath the

stand. The samples were moved back and forth under the laser light until a diffraction pattern was observed. The origin and the two diffraction bands were recorded on the white paper. Five sarcomeres per sample were determined, and sarcomere length (μ) was determined by the equation provided by Cross et al. (1981):

Sarcomere length (μm) =

$$\frac{0.6328 \text{ X D}\sqrt{(\frac{\text{T}}{\text{D}})^2 + 1}}{\text{T}}$$

D = distance from specimen to diffraction pattern screen in mm (preferably 100 mm)

T = spacing between diffraction bands in mm

0.6328 = 632.8 (the wavelength of the laser) x $10^{-3}$

**Myofibrillar protein isolation (Appendix III).** Myofibrillar proteins were isolated using the procedure described by Pietrzak et al. (1997) with modifications. Three grams of powdered meat sample were suspended in ice-cold 10 mL rigor buffer (0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 10 mM $K_2HPO_4$) at pH 7.4 and homogenized using a Polytron homogenizer (model CH-6010; Kinematica) at setting 6 for 15 s. The homogenate was filtered thorough doubled-layered cheese cloth to remove connective tissue and fat. One milliliter of homogenate was transferred and centrifuged at 4,000 x *g* for 5 min. The supernatant was decanted and the pellet was resuspended in 1 mL of ice-cold rigor buffer. The pellet washing step was repeated three times. One milliliter of extraction buffer (0.1 M Tris-HCl, 1.25 mM EDTA, 5% SDS) at pH 8 was added to the washed pellet and

vortexed thoroughly. The sample was left in room temperature for 5 min and centrifuged at 4,000 x *g* for 5 min.

**Protein concentration (Appendix IV).** One hundred microliters of myofibrillar protein samples were transferred to a new tube and diluted with 900 µL of extraction buffer. Protein concentration was determined using a Pierce bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL). A concentration series (20 – 2,000 µg/mL) of bovine serum albumin (BSA) standards were prepared using the extraction buffer as the diluent. Twenty five microliters of BSA standards and diluted myofibrillar protein samples were deposited into their respective wells on a 96 wells microplate (Microtest III sterile 96-well flat-bottomed microplate; Becton Dickinson & Company). Two hundred microliters BCA working reagents (50:1 of Reagent A: Reagent B; Pierce Biotechnology) were added to each respective well on the microplate and incubated at 37$^{o}$C for 30 min. After cooling to the room temperature, absorbance was read at 562 nm, and protein concentrations were expressed as µg/mL. All myofibrillar protein samples were diluted to 2 mg/mL with dd$H_2O$.

**Gel electrophoresis (Appendix V).** Degree of proteolysis was measured by troponin-T degradation. All of the following procedures were conducted at room temperature. Twenty-five microliters of the 2 mg/mL myofibrillar protein samples were mixed with 2x Laemmli sample buffer (65.8 mM Tris-HCl, 2.1% SDS, 26.3% glycerol, 0.01% bromophenol blue) with 5% β-mercaptoethanol at 1:1 ratio. All samples were heated at 95°C for 5 min. Five microliters Kaleidoscope Pre-stained Protein Standard and prepared

myofibrillar protein samples (5 μg) were loaded on 4-20% Mini-PROTEAN TGX™ precast polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) using a Bio-Rad Mini-PROTEIN 2 Cells system (Bio-Rad Laboratories). The system was run at constant voltage of 200 V for 40 min with a running buffer consisted of 25 mM Tris-base, 192 mM glycine and 0.1% SDS (pH 8.3).

**Western Blotting (Appendix VI)**. Proteins in the gels were blotted to polyvinylidene difluoride (PVDF) membranes (0.45 μm; Immobilon-FL transfer membrane; Millipore) using a Bio-Rad Mini-Trans-Blot Electrophoretic transfer cell (Bio-Rad Laboratories) for 60 min at a constant voltage of 100 V with ice-cold transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol, 0.1% SDS at pH 9.2). Membranes were blocked for 2 hr in Odyssey Blocking Buffer (LI-COR, Lincoln, NE) and incubated in primary anti-troponin-T (JLT-12; Sigma-Aldrich, St. Louis, MO) antibody at a dilution of 1:10,000 in Odyssey blocking buffer containing 0.2% Tween-20 for 1 h. Membranes were washed 3 times with Tris Buffered Saline containing 0.2% Tween-20 for 15 min and incubated in IRDye 680LT Conjugated Goat Anti-Mouse IgG1 (LI-COR) secondary antibody at a dilution of 1:10,000 in Odyssey blocking buffer containing 0.2% Tween-20 for 1 h. Membranes were washed 3 times and scanned using Odyssey Infrared Imaging system (LI-COR) at 700 nm. All intact troponin-T and degraded troponin-T products were measured by quantifying band intensities (k. pixels) using Odyssey application software version 1.1. Bands 1 and 2 (38 and 35 kD, respectively) corresponded to intact troponin-T while bands 3 and 4 (30 and 28 kD, respectively) correspond to degraded troponin-T.

Percent troponin-T degraded was measured by band intensities of degraded bands divided by band intensities of all bands in a specific lane.

**Sarcoplasmic reticulum (SR) membrane extraction (Appendix VII). :** The SR membrane was extracted using the procedure described by Hemmings (2001). Ten g of powdered samples were suspended in ice-cold 35 mL homogenization buffer (10mM $NaHCO_3$, 2 mM sodium azide, 10 mM Tris-Cl, and 1 mM dithiothreitol) at pH 7.5 and homogenized using a Polytron homogenizer (Kinematica) at setting 6 for 15 s. Homogenate was transferred into a 50 mL plastic centrifuge tube and centrifuged at 2,000 × *g* for 10 min at 4$^{o}$C. The supernatant was collected and centrifuged at 10,000 x *g* (Sorvall RC5B Superspeed Centrifuge; Thermo Scientific, Rockford, IL) for 30 min at 4$^{o}$C. Four and a half percent of KCl (w/v) was added to the supernatant and stirred for 30 min on ice. The supernatant was centrifuged at 100,000 x *g* for 60 min at 4$^{o}$C (Beckman L7-65 Ultracentrifuge with a SW28 rotor; Beckman Coulter). The final supernatant was discarded, and the pellet was resuspended in 1 mL of 10mM tris buffer and stored at -80$^{o}$C until use.

**SR membrane lipid extraction (Appendix VIII)**. The SR membrane lipid was extracted following the procedure described by Bligh and Dyer (1959) with modifications. Three and seventy five hundredths milliliters of 1:2 chloroform: methanol was added to the SR membrane and tris buffer mixture from the previous extraction. The tube was vortexed for 5 s, mixed on a shaker for 20 min and stored overnight at 4$^{o}$C. The next morning, 1.25 mL of chloroform was added, and the sample was mixed on a shaker for one min.

One and twenty five hundredths milliliters of $ddH_2O$ was added, and the sample was mixed again on a shaker for another min. The homogenate was filtered through a Whatman #2 filter paper (Whatman, Clifton, NJ) into 13 x 100 mm screw cap tube. Samples were centrifuged at 1,000 x *g* for 5 min. Following centrifugation, the aqueous phase (top layer) was removed with a Pasteur pipet. The remaining was evaporated to dryness under nitrogen at 60°C.

**SR membrane fatty acids (IX).** After extraction, lipids were converted to fatty acid methyl esters according to the procedures by Morrison and Smith (1964) and Metcalfe et al. (1966). One half milliliters of 0.5 M NaOH in methanol was added to the extracted SR membrane lipid, and the solution was vortexed for 5 sec and heated for 5 min at 100°C. One half milliliters of boron trifluoride in 14% methanol was added into the solution, and the solution was vortexed for 5 s and heated for 5 min at 100°C. One milliliter of a saturated NaCl solution and 1 mL of hexane were added to the solution, and the solution was vortexed for 5 s. The solution was centrifuged at 1,000 x g for 5 min. Following centrifugation, hexane layer (top layer) was removed and placed in a gas chromatography (GC) vial. The prepared fatty acid methyl esters were further concentrated by drying at 60°C under constant nitrogen gas purging and mixed with 100 µL of hexane. The fatty acid methyl esters were transferred to 100 µL spring bottom vial inserts and inserted into the GC vials. The fatty acids were analyzed using gas chromatography (Hewlett-Packard 6890 FID GC System; Agilent Technologies, Santa Clara, CA) with a capillary column (Chrompack CP-Sil 88 (0.25 mm x 100 m). Oven temperature was programmed from 140 to 220°C at 2°C/min and held at 220°C for 20 min. Injector and detector temperature

was maintained at 270 and 300°C, respectively. The carrier gas Helium had a flow rate of 30 mL/min. Fatty acid classes were identified by comparison of retention times with known standards.

**SR membrane phospholipids, neutral lipids and total lipids (Appendix X).** A second set of SR membrane lipid was extracted. Thirty microliters of 2% methanol and 1 % dd$H_2O$ in chloroform was added to each lipid sample. For SR membrane phospholipids, neutral lipid and total lipid profile, samples were separated into 10 different lipid groups by one-dimensional thin-layer chromatography (TLC) described by Leray et al. (1987) with modifications. Whatman LK5 TLC plates (Whatman) were prewashed by migration up to 1 cm from the top with chloroform/methanol (1/1, v/v) and wetted thoroughly with 2.3% (w/v) boric acid solution. All 30 μL of each SR lipid sample was deposited on the plate and placed in the chromatography tank containing the following solvent: chloroform/ethanol/dd$H_2O$/triethylamine (30/35/7/35, v/v). After the migration was complete, lipid and phospholipid spots were quantified by the method described by Baron and Coburn (1984) with modification. Each plate was dampened with a 10 % (w/v) cupric sulfate solution in 8% (w/v) phosphoric acid and placed in an oven at 180°C for 10-15 min. The isolated fractions were identified by comparing their $R_f$ values with known lipid standards. The plate was scanned by a desktop scanner (Artisan 730, Epson, Nagano, Japan) and the isolated fractions were quantified using Quantity One 1-D Analysis Software (Bio-Rad). Each phospholipid or neutral lipid was measured as a percentage of total phospholipids or neutral lipid in one lane. For total lipid profiling, all phospholipids and all neutral lipids from one lane were combined as one class. Each lipid

class was measured as a percentage of total lipids on one specific lane.

**Statistical analysis**. Data for WBSF, free calcium concentration, TBA, and troponin-T degradation were analyzed as a split-split plot design with dietary treatments as the whole-plot, aging period as the split-plot and retail display time as the split-split plot. The experimental unit was the individual animal. Sarcomere length, SR membrane fatty acid, phospholipid and total lipid profiles were analyzed as a completely randomized design. Data were analyzed using the GLIMMIX procedure of SAS (version 9.2, Cary, NC, 2009). Separation of means was conducted using LSMEANS procedure with PDIFF or SLICEDIFF options at $P < 0.05$.

**Manuscript 2 - Effects of dietary antioxidant supplementation of cattle finished with 30% wet distillers grains plus solubles on fatty acid profiles and display life**

Samples for manuscript 2 and manuscript 3 were from the same set of animals under one sample collection and preparation.

**Animals.** One hundred and sixty Continental X British cattle were blocked by BW, stratified by BW within each block, and assigned randomly to pens within block. Pens were randomly assigned to one of the eight treatments with two pens per treatment and ten cattle per pen. Cattle were fed for 106 d on either a corn-only diet or a corn-based diet with 30% WDGS (DM basis) with four antioxidant treatments. The eight treatments were 1) a corn-only diet with E supplementation at 22.5mg (50 IU)/hd/d (control); 2) a corn-only diet with E supplementation at 450 mg (1,000 IU)/hd/d (Corn+E); 3) a corn-only

diet with AG supplementation at 3 g/hd/d (215 mg/kg of feed) (Corn+AG); 4) a corn-only diet with a combination of 225 mg (500 IU)/hd/d of E and 3 g/hd/d (215 mg/kg of feed) of AG (Corn +E+AG); 5) a corn based diet with 30% WDGS with E supplementation at 22.5 mg (50 IU)/hd/d (WDGS); 6) a corn based diet with 30% WDGS with E supplementation at 450 mg (1,000 IU)/hd/d (WDGS+E); 7) a corn based diet with 30% WDGS with AG supplementation at 3 g/hd/d (215 mg/kg of feed) (WDGS+AG); 8) a corn based diet with 30% WDGS with a combination of 225 mg (500 IU)/hd/d of E and 3 g/hd/d (215 mg/kg of feed) of AG (WDGS+E+AG).

**Sample collection and preparation.** All cattle were harvested at a commercial abattoir (Greater Omaha Packing Co., Omaha, NE) on January 28, 2014. After 48 h of postmortem chilling, 80 carcasses (5 carcasses per pen) were selected (10 carcasses from each treatment). Carcass selection was based on treatment and quality grade (Choice). The strip loins were collected, vacuumed-packaged and transported to Loeffel meat laboratory at the University of Nebraska-Lincoln. Strip loins from the left sides of the carcasses were fabricated on d 2 and 7 of postmortem aging, and strip loins from the right sides of carcasses were fabricated on d 14 of postmortem aging. Each strip loin was fabricated into 2 tenderness samples (2.54 cm) and 3 lab samples (1.27 cm) for each aging period from the anterior to the posterior end of the loin muscle. The remainders of the strip loins were immediately vacuum packaged (3 mm STD barrier, Prime Sources, St. Louis, OM) on a Multivac Packaging machine (MULTIVAC C500, Multivac Inc., Kansas city, MO) and aged to the next designated aging period. Upon fabrication, steaks were overwrapped with oxygen permeable polyvinyl chloride film (PSM18, Prime

Source, St. Louis, MO) and subjected to retail display conditions (2 ± 2°C, and exposed to continuous 1,000-1,800 lux warm white fluorescence lighting). For each aging period, samples for tenderness, free calcium concentrations and proteolysis were obtained on d 0 and 7 of retail display (manuscript 3), and samples for lipid oxidation (manuscript 2) assessments were obtained on d 0, 4 and 7 of retail display. Tenderness samples allotted for d 7 retail display were used to evaluate daily objective color and subjective discoloration scores during retail display period. Muscle tissue fatty acids profile, E and ethoxyquin concentration samples (manuscript 2) were obtained on d 0 of retail display after 14 d of aging. Sarcomere length (manuscript 3) was determined on samples with 0 d retail display after 2 d of aging. For SR membrane fatty acid, phospholipid and lipid analyses (manuscript 3), samples were also obtained at d 0 of retail display after 14 d of aging. At the end of the allotted treatments, all samples were vacuum packaged and frozen at -20°C (tenderness samples) or -80°C (lab samples) until analyzed. The lab samples were pulverized in liquid nitrogen with a blender (model 51BL32, Waring Commercial, Torring, CT).

**Discoloration.** A five-person trained panel consisting of graduate students in the Animal Science Department at the University of Nebraska-Lincoln subjectively evaluated discoloration of each steak as a percentage (0 – 100%) of total surface area. Panelists were trained using a system of open discussion and a range of discoloration.

**Objective color.** Objective color measurements were obtained for CIE L*, a*, and b* values using a Minolta CR-400 colorimeter (Minolta, Osaka, Japan) set at a D65 light

source and 2° observer with an 8 mm diameter measurement area. The colorimeter was calibrated daily using a white ceramic tile provided by the manufacturer, and color measures were obtained at 0,1, 2, 3, 4, 5, 6 and 7 d of display by averaging 6 reading from different areas of the steak surface. The CIE a* measurements, a measure of red to green, were used to determine color stability of the steaks.

**Muscle tissue lipid extraction.** Total muscle tissue lipid was extracted following the procedure described by Folch et al. (1957). Ten milliliters of 2:1 chloroform: methanol was added to a chloroform resistant tube with the 1 g of pulverized muscle tissue. The tube was vortexed for 5 s and stood for 1 hr at room temperature. The homogenate was filtered through Whatman #2 filter paper (Whatman) into 13 x 150 mm screw cap tube bringing the final volume with 2:1 chloroform: methanol to 10 mL. Two milliliters a 0.74% KCl solution was added, and mixture was vortexed for 5 s. Samples were centrifuged at 1,000 x *g* for 5 min. Following centrifugation, the aqueous phase (top layer) was removed with a Pasteur pipet. The remaining layer was evaporated to dryness under nitrogen at 60°C.

**Muscle tissue fatty acids.** Muscle tissue fatty acids were determined from the muscle tissue lipid extraction using the same methodology as described in SR membrane fatty acids section of manuscript 1 without concentrating and transferring the fatty acids to 100 µL spring bottom vial inserts.

**Lipid oxidation.** Lipid oxidation was determined using the same methodology as described in lipid oxidation section of manuscript 1.

**Vitamin E extraction.** Vitamin E level in muscle tissue was quantified by the procedure described by Nierenberg and Nann (1992) with modifications. One gram of powdered meat sample was combined with 3 mL of 6% pyrogallol and 1 mL of saturated potassium hydroxide. The mixture was digested for 30 min at 70$^{o}$ C, and 5 mL of dd$H_2O$ and 1 mL of hexane containing 0.05% BHT was added after cooling. The mixture was centrifuged at 10,000 x *g* for 2 min, and the supernatant was collected. The extraction process was repeated for 3 times. Pooled supernatants were evaporated to dryness under nitrogen flush, and the residue was resuspended in 200 µL of tetrahydrofuran. The sample was brought to 300 µL with mobile phase. The mobile phase consisted of acetonitrile: tetrahydrofuran: methanol: 1% ammonium acetate in dd$H_2O$ (65:25:6:4, v/v/v/v) containing 0.1% BHT and 0.05% trimethylamine. Fifty microliters of samples were injected into a high performance liquid chromatography (HPLC) system (Waters Associates instruments; 600E solvent delivery system and 2487 UV detector, Milford, MA) with a reversed phase Microsorb-MV100 C18 column (5 µm, 250 × 4.6 mm; Rainin Instruments, Woburn, MA). The flow rate was set at 1.0 mL/min and absorbance was read at 292 nm for E.

**Ethoxyquin extraction.** Ethoxyquin in muscle tissue was extracted by the method b extraction procedure described by Aoki et al. (2010). Five grams of powdered meat samples were combined with 20 mL of methanol and 10 g of sodium sulfate anhydride

and homogenized using a Polytron homogenizer (Kinimatica) at low speed setting. The homogenate was centrifuged at 4,000 x *g* for 5 min, and the supernatant was collected. The extraction procedure was repeated one more time, and the supernatant was combined. Ten milliliters of one-propanol was added to the extraction solution to prevent bumping and the solution was evaporated to dryness. The residue was dissolved in 5 mL of acetonitrile: water (2:3, v/v), sonicated, and filtered through a 0.45 μM membrane filter (Rainin Instruments). Five microliters filtered sample solution was injected into HPLC system (Waters Associates instruments; 600E solvent delivery system, and 474 florescence detector). The mobile phase consisted of acetonitrile: 0.01M ammonium acetate (4:1 v/v). The flow rate was set at 0.2 mL/min, and the excitation and emission wavelengths for fluorescence monitoring of ethoxyquin were 360 and 436 nM, respectively.

**Statistical analysis.** Data for TBA were analyzed as a split-split plot design with dietary treatments as the whole-plot, aging period as the split-plot and retail display time as the split-split plot. Color data were analyzed as a split-split-plot repeated measures design with dietary treatments as the whole-plot, aging period as the sub-plot and retail display days as the repeated measures. The experimental unit was the individual animal. The E and ethoxyquin concentrations and fatty acids profile were analyzed as a completely randomized design. Data were analyzed using the GLIMMIX procedure of SAS (version 9.2, Cary, NC, 2009). Separation of means was conducted using LSMEANS procedure with PDIFF or SLICEDIFF options at $P < 0.05$. In addition, the CONTRAST statements

in SAS were used to compare effects of feeding Corn vs. WDGS, E vs. no E, AG vs. no AG and E+AG vs. no E+AG.

**Manuscript 3 - Feeding vitamin E may reverse sarcoplasmic reticulum membrane instability caused by feeding wet distillers grains plus solubles to cattle**

Samples for manuscript 2 and manuscript 3 were from the same set of animals under one sample collection and preparation. Free calcium concentration, WBSF, sarcomere length, proteolysis, SR membrane extraction, SR membrane lipid extraction, SR membrane fatty acids, SR membrane phospholipid, neutral lipid and total lipid were all performed using the same methodologies as described in manuscript 1.

**Statistical Analysis.** Data for free calcium concentration and TNT degradation were analyzed as a split-split plot design with dietary treatments as the whole-plot, aging period as the split-plot and retail display time as the split-split plot. The experimental unit was the individual animal. Sarcomere length, SR membrane fatty acid, phospholipid, neutral lipid and total lipid profiles were analyzed as a completely randomized design. Data were analyzed using the GLIMMIX procedure of SAS (version 9.2, Cary, NC, 2009). Separation of means was conducted using LSMEANS procedure with PDIFF or SLICEDIFF options at $P < 0.05$. In addition, the CONTRAST statements in SAS were used to compare effects of feeding Corn vs. WDGS, E vs. no E, AG vs. no AG and E+AG vs. no E+AG.

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Running Head: SR membrane instability

# Feeding wet distillers grains plus solubles contributes to sarcoplasmic reticulum membrane instability

**M. D. Chao, K. I. Domenech-Perez, L. S. Senaratne-Lenagala, and C. R. Calkins[1]**

Department of Animal Science, University of Nebraska-Lincoln, Lincoln, NE 68583-0908

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[1]Corresponding author: Ccalkins1@unl.edu

**ABSTRACT:** It is well-known that feeding high levels of wet distillers grains plus solubles (WDGS) increases polyunsaturated fatty acid (PUFA) levels in beef. Perhaps, WDGS in feedlot diets increases PUFA concentration in the sarcoplasmic reticulum (SR) membrane, thereby altering membrane integrity, resulting in more rapid calcium leakage and improved tenderness. The objective of this study was to evaluate this hypothesis. A total of 96 steers were used in a randomized complete block design with two treatments. Steers were fed either a corn-only diet or a corn-based diet with 50% WDGS. Fifteen striploins (*Longissimus lumborum*) from each treatment (n=30) were collected and aged for 2, 7, 14, or 21 d. Steaks were removed at each aging period and placed under retail display conditions for 0, 4, and 7 d. Steaks were used to measure tenderness, proteolysis, free calcium concentrations, lipid oxidation, sarcomere length and SR membrane fatty acid, phospholipid lipid, neutral lipid and total lipid profiles. Compared to steaks from steers fed corn-only, steaks from steers fed WDGS were more tender ($P < 0.05$) and had higher ($P < 0.05$) free calcium concentrations at 2 d aging. Feeding WDGS increased ($P < 0.05$) C18:0 and C18:2 and tended to increase ($P < 0.1$) total PUFA concentrations in the SR membrane. Feeding WDGS also decreased ($P < 0.05$) C15:1, C16:1, C17:1, C18:1, C18:1V and total monounsaturated fatty acids in the SR membrane. Furthermore, feeding WDGS tended to decrease ($P < 0.10$) total phospholipid concentration and tended to increase ($P < 0.10$) total neutral lipid concentration for SR membrane lipid profile. Finally, feeding WDGS increased ($P < 0.01$) phosphatidylcholine, but decreased ($P < 0.05$) phosphatidylethanolamine percentages for SR membrane phospholipid profile. There were no differences ($P > 0.10$) between treatments for sarcomere length. Although differences in tenderness between the two treatments were detected, there were no

corresponding differences in proteolysis. Steaks from steers fed corn-only had higher ($P < 0.05$) lipid oxidation values compared to steaks from steers fed WDGS at 21 d aging. This study confirmed that feeding WDGS can increase tenderness and free calcium release early postmortem. However, the mechanism that contributes to SR membrane instability is still unclear.

Key words: Beef, distillers grains, sarcoplasmic reticulum, fatty acids

## INTRODUCTION

Tenderness has repeatedly been cited as the most important element for both eating quality and consumer purchasing decisions (Miller et al., 2001; Platter et al., 2005) and is a high priority for research in the meat industry (Boleman et al., 1998; Garcia et al., 2008; McKenna et al., 2002). The mechanism of meat tenderization is a well understood subject. However, the way animal diets might affect the basic mechanism of meat tenderization requires further exploration.

When compared to corn, distillers grains are not only less expensive, but also contain up to three times the levels of protein, fiber, and fat (Klopfenstein et al., 2007). Hence, distillers grains have been used widely in feedlot diets at levels varying from 10 to 80% on a dry matter basis. Although many studies (Koger et al., 2010; Mello et al., 2012; Roeber et al., 2005) on beef quality from feeding distillers grains showed no differences in tenderness, a recent study (Senaratne, 2012) revealed an intriguing phenomenon. Beef from steers fed WDGS was more tender than beef from steers fed corn-only. This is not the first time that feeding WDGS has been reported to improve

tenderness (Depenbusch et al., 2009; Segers et al., 2011). The mechanism of this increased tenderization was the subject of this research.

The hypothesis was that PUFA content of the sarcoplasmic reticulum (SR) membrane would increase as a consequence of feeding WDGS, which would predispose the SR membrane to release calcium earlier than normal as a result of rapid membrane oxidation, causing an early activation of calcium dependent proteases (the calpain system) and thereby enhance tenderness. The objective of this study was to evaluate this hypothesis through examination of tenderness, lipid oxidation, sarcomere length and proteolysis during aging and retail display as well as changes in SR membrane fatty acid, phospholipid, neutral lipid and total lipid profiles of beef with varying degrees of oxidation capacity.

## MATERIALS AND METHODS

All animal use protocols were approved by the University of Nebraska-Lincoln's Animal Care and Use Committee.

### *Animals*

A total of 96 steers were used in a randomized complete block design with 2 treatments. Steers were fed for 147 d on either a corn-only diet or a corn-based diet with 50% WDGS (DM basis). Steers were also implanted on d 1 with Revelor-XS (Merck Animal Health, Summit, NJ). Animals were blocked by BW, stratified by BW within each block, and assigned randomly to pens. Pens were randomly assigned to 1 of the 2 treatments with 6 pens per treatment and 8 steers per pen.

*Sample Collection, Fabrication and Preparation*

All steers were harvested at a commercial abattoir. After 48 h of postmortem chilling, 30 out of 96 carcasses were selected (15 carcasses from each treatment). Carcass selection was based on treatment and quality grade (low Choice). Strip loins (*Longissimus lumborum*) from all selected carcasses were collected, vacuumed-packaged and transported to Loeffel meat laboratory at the University of Nebraska-Lincoln.

Strip loins from the left sides of the carcasses were fabricated on postmortem aging d 2 and 7, and strip loins from the right sides of carcasses were fabricated on postmortem aging d 14 and 21. Each strip loin was fabricated into 2 tenderness samples (2.54 cm) and 3 lab samples (1.27 cm) for each aging period, from the anterior to the posterior end of the loin muscle. The remainder of the strip loins were immediately vacuum packaged in vacuum pouches (3 mm STD barrier, Prime Source, St. Louis, MO) on a Multivac Packaging machine (Mutivac C500, Multivac, Kansas city, MO) and aged to the next designated aging period.

Upon fabrication, steaks subjected to retail display conditions (2 ± 2$^{o}$C, and exposed to continuous 1,000-1,800 lux warm white fluorescence lighting) were packaged in Styrofoam trays (Styro-Tech, Denver, CO) and overwrapped with oxygen permeable polyvinyl chloride film (PSM18, Prime Source, St. Louis, MO). Samples for objective tenderness, free calcium concentrations and proteolysis were obtained on d 0 and 7 of retail display for each aging period, and samples for lipid oxidation were obtained on d 0, 4 and 7 of retail display for each aging period. Sarcomere length was determined on samples with 0 d retail display after 2 d of aging. For SR membrane fatty acid, phospholipid, neutral lipid and total lipid analyses, samples were obtained at d 0 of retail

display after 14 d of aging. Samples were vacuum packaged and frozen at -20°C (tenderness samples) or -80°C (lab samples) until analysis. The lab samples were pulverized in liquid nitrogen with a blender (model 51BL32, Waring Commercial, Torring, CT).

*Objective Tenderness (WBSF)*

Steaks were removed from the freezer and thawed at 4°C for 24 h prior to grilling. An insulated type T thermocouple (5SC-TT-T-30-120, OMEGA Engineering, Inc., Stamford, CT) was inserted into the geometric center of each steak and attached to an OMEGA 450-ATT thermometer (OMEGA Engineering, Inc., Stamford, CT) to monitor the internal temperature of the steak. Steaks were grilled on a Hamilton Beach Indoor-Outdoor grill (Model 31605A, Proctor-Silex, Inc., Washington, NC), flipped once when the internal temperature reached 35°C, and removed from the grill when they reached an internal temperature of 71°C. Grilled steaks were cooled at 4°C for 24 h, and six cores, 1.27 cm in diameter, were removed parallel to the muscle fibers using a drill press. Cores were sheared on a Texture Analyzer (model TMS-PRO, Food Technology Crop., Sterling, VA) with a Warner-Bratzler blade. The mean peak shear force (kg) of 6 cores was calculated for each steak.

*Free Calcium Concentration*

Free calcium was quantified using the procedure described by Parrish et al. (1981) with modifications. Three grams of powdered sample were centrifuged at 196,000 x *g* (Beckman L7-65 Ultracentrifuge with a SW55Ti rotor; Beckman Coulter, Brea, CA) at 4°

C for 30 min. Seven hundred microliters of the supernatant was collected and treated with 0.1 mL of 27.5% trichloroacetic acid (TCA). Samples were centrifuged at 6,000 x g (Eppendorf model 5430; Eppendorf, Hamburg, Germany) for 10 min. Four hundred microliters of supernatant was transferred to a syringe, and the volume was brought to 4 mL with deionized distilled water ($ddH_2O$). The diluted calcium sample was filtered through 13 mm diameter Millex-LG 0.20 μm syringe filters (Millipore, Bedford, MA). Calcium concentration of samples was quantified using an inductively-coupled plasma emission spectrometer (iCAP 6500 Radial; Thermo Electron, Cambridge, UK) with appropriate calcium concentration standards.

*Lipid Oxidation*

Lipid oxidation samples were prepared using the thiobarbituric acid assay (TBA) described by Ahn et al. (1998). Duplicate 200 μL aliquots of supernatant from each prepared sample were transferred to 96-well plates and read with a microplate spectrophotometer (Model Epoch, Biotek, Winooski, VT) at 540 nm. All 96-well plates had standards to calculate standard curves, and each sample was calculated as mg of malonaldehyde per kg of tissue using the standard curve from each plate.

*Sarcomere Length*

Sarcomere length was determined using the powdered sample Helium-Neon laser method described by Cross et al. (1981) with modifications from Dolazza and Lorenzen (2014). Five sarcomeres per sample were determined, and the final sarcomere length was calculated according to the equation described by Cross et al. (1981).

*Proteolysis*

Myofibrillar proteins were isolated according the procedure described by Pietrzak et al. (1997) with modifications. Three grams of powdered meat sample were suspended in ice-cold 10 mL rigor buffer (0.1 *M* KCl, 2 m*M* $MgCl_2$, 1 m*M* EGTA, and 10 m*M* $K_2HPO_4$) at pH 7.4 and homogenized using a Polytron homogenizer (model CH-6010; Kinematica, Luzern, Switzerland) at setting 6 for 15 s. The homogenate was filtered thorough doubled-layered cheese cloth to remove connective tissue and fat. One milliliter of homogenate was transferred and centrifuged at 4,000 x *g* for 5 min. The supernatant was decanted and the pellet was resuspended in 1 mL of ice-cold rigor buffer. The pellet washing step was repeated three times. One milliliter of extraction buffer (0.1 M Tris-HCl, 1.25 m*M* EDTA, 5% SDS) at pH 8 was added to the washed pellet and vortexed thoroughly. Protein concentration was determined using a Pierce bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL). All myofibrillar protein samples were diluted to 2 mg/mL with $ddH_2O$.

Degree of proteolysis was measured by troponin-T degradation. All of the following procedures were conducted at room temperature. Twenty-five microliters of the 2 mg/mL myofibrillar protein samples were mixed with 2x Laemmli sample buffer (65.8 m*M* Tris-HCl, 2.1% SDS, 26.3% glycerol, 0.01% bromophenol blue) with 5% β-mercaptoethanol at 1:1 ratio. All samples were heated at 95°C for 5 min. Five microliters Kaleidoscope Pre-stained Protein Standard and prepared myofibrillar protein samples (5 µg) were loaded on 4-20% Mini-PROTEAN TGX™ precast polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) using a Bio-Rad Mini-PROTEIN 2 Cells system (Bio-Rad Laboratories). The system was run at constant voltage of 200 V for 40 min with a

running buffer consisted of 25 m*M* Tris-base, 192 m*M* glycine and 0.1% SDS (pH 8.3). Proteins in the gels were blotted to polyvinylidene difluoride (PVDF) membranes (0.45 μm, Immobilon-FL transfer membrane; Millipore) using a Bio-Rad Mini-Trans-Blot Electrophoretic transfer cell (Bio-Rad Laboratories) for 60 min at a constant voltage of 100 V with ice-cold transfer buffer (25 m*M* Tris-base, 192 m*M* glycine, 20% methanol, 0.1% SDS at pH 9.2). Membranes were blocked for 2 hr in Odyssey Blocking Buffer (LI-COR, Lincoln, NE) and incubated in primary anti-troponin-T (JLT-12; Sigma-Aldrich, St. Louis, MO) antibody at a dilution of 1:10,000 in Odyssey blocking buffer containing 0.2% Tween-20 for 1 h. Membranes were washed 3 times with Tris Buffered Saline containing 0.2% Tween-20 for 15 min and incubated in IRDye 680LT Conjugated Goat Anti-Mouse IgG1 (LI-COR) secondary antibody at a dilution of 1:10,000 in Odyssey blocking buffer containing 0.2% Tween-20 for 1 h. Membranes were washed 3 times and scanned using Odyssey Infrared Imaging system (LI-COR) at 700 nm.

All intact troponin-T and degraded troponin-T products were measured by quantifying band intensities (k. pixels) using Odyssey application software version 1.1. Bands 1 and 2 (38 and 35 kD, respectively) corresponded to intact troponin-T while bands 3 and 4 (30 and 28 kD, respectively) correspond to degraded troponin-T. Percent troponin-T degraded was measured by band intensities of degraded bands divided by band intensities of all bands in a specific lane.

*SR Membrane Extraction*

The SR membrane was extracted according to the procedure described by Hemmings (2001) with modifications. Ten grams of powdered samples were suspended

in ice-cold 35 mL homogenization buffer (10 m*M* $NaHCO_3$, 2 m*M* sodium azide, 10 m*M* Tris-Cl, and 1 m*M* dithiothreitol) at pH 7.5 and homogenized using a Polytron homogenizer (Kinematica) at setting 6 for 15 s. Homogenate was transferred into a 50 mL plastic centrifuge tube and centrifuged at 2,000 × *g* for 10 min at 4°C. The supernatant was collected and centrifuged at 10,000 x *g* (Sorvall RC5B Superspeed Centrifuge; Thermo Scientific, Rockford, IL) for 30 min at 4°C. Four and a half percent of KCl (w/v) was added to the supernatant and stirred for 30 min on ice. The supernatant was centrifuged at 100,000 x *g* for 60 min at 4°C (Beckman L7-65 Ultracentrifuge with a SW28 rotor; Beckman Coulter). The final supernatant was discarded, and the pellet was resuspended in 1 mL of 10mM tris buffer and stored in -80°C until use.

*SR Membrane Fatty Acids*

For SR membrane fatty acids, total lipid was extracted following the procedure by Bligh and Dyer (1959). After extraction, lipids were converted to fatty acid methyl esters according to the procedures by Morrison and Smith (1964) and Metcalfe et al. (1966). The prepared fatty acid methyl esters were further concentrated by drying at 60°C under constant nitrogen gas purging and mixed with 100 µL of hexane. The fatty acid methyl esters were transferred to 100 µL spring-bottom vial inserts and inserted into the GC vials. The samples were analyzed using gas chromatography (Hewlett-Packard 6890 FID GC System; Agilent Technologies, Santa Clara, CA) with setups described by Mello et al. (2012).

*SR Membrane Phospholipids, Neutral Lipid and Total Lipids*

Total lipids were extracted from a second set of SR membrane samples. Thirty microliters of 2% methanol and 1 % dd$H_2O$ in chloroform was added to each lipid sample. For SR membrane phospholipids, neutral lipid and total lipid profile, samples were separated into 10 different lipid groups by one-dimensional thin-layer chromatography (TLC) described by Leray et al. (1987) with modifications. Whatman LK5 TLC plates (Whatman, Clifton, New Jersey) were prewashed by migration up to 1 cm from the top with chloroform/methanol (1/1, v/v) and wetted thoroughly with 2.3% (w/v) boric acid solution. All 30 µL of each extracted lipid sample was deposited on the plate and placed in the chromatography tank containing the following solvent: chloroform/ethanol/dd$H_2O$/triethylamine (30/35/7/35, v/v). After the migration was complete, lipid and phospholipid spots were quantified by the method described by Baron and Coburn (1984) with modification. Each plate was dampened with a 10 % (w/v) cupric sulfate solution in 8% (w/v) phosphoric acid and placed in an oven at 180°C for 10-15 min. The isolated fractions were identified by comparing their $R_f$ values with known lipid standards. The plate was scanned by a desktop scanner (Artisan 730, Epson, Nagano, Japan) and the isolated fractions were quantified using Quantity One 1-D Analysis Software (Bio-Rad). Each phospholipid was measured as a percentage of total phospholipids, and each neutral lipid was measured as a percentage of total neutral lipids. For total lipid profiling, all phospholipids were combined as one class, and all neutral lipids were combined as one class. Each lipid class was measured as a percentage of total lipids on one specific lane.

*Statistical Analysis*

Data for WBSF, free calcium concentration, TBA, and troponin-T degradation were analyzed as a split-split plot design with dietary treatments as the whole-plot, aging period as the split-plot and retail display time as the split-split plot. Sarcomere length, SR membrane fatty acid, phospholipid and total lipid profiles were analyzed as a completely randomized design. Data were analyzed using the GLIMMIX procedure of SAS (version 9.2, Cary, NC, 2009). Separation of means was conducted using LSMEANS procedure with PDIFF or SLICEDIFF options at $P < 0.05$.

## RESULTS

Results for the SR membrane fatty acid profile analyses are shown in Table 1. Feeding WDGS decreased ($P < 0.05$) concentrations of 15:1, 16:1, 17:1, 18:1, 18:1V and total monounsaturated fatty acids (MUFA), increased ($P < 0.05$) concentrations of 18:0 and 18:2 fatty acids and tended to increase ($P < 0.10$) total PUFA in the SR membrane. Long and very long chain PUFAs like 20:3, 20:4, 20:5, 22:4, 22:5 were not affected by the treatment. Results for the SR membrane phospholipids, neutral lipid and total lipid profiles are shown in Table 2. Feeding WDGS tended to decrease ($P < 0.10$) phospholipid concentration and tended to increase ($P < 0.10$) total neutral lipid concentrations in the SR membrane. Feeding WDGS also increased ($P < 0.01$) concentration of phosphatidylcholine (PC), but decreased ($P < 0.05$) concentration of phosphatidylethanolamine (PE) in the SR membrane phospholipids.

There was a significant ($P < 0.05$) three-way interaction among treatment, aging and retail display time on steak WBSF. Compared to steaks from steers fed corn-only,

steaks from steers fed 50% WDGS were more tender ($P < 0.01$) at 2 d of aging with 0 day of retail display (Figure 1). There was also a significant ($P < 0.05$) three-way interaction among treatment, aging and retail display on steak free calcium. Steaks from WDGS-fed steers had higher ($P < 0.01$) free calcium concentrations at 2 d aging after 7 d of retail display (Figure 2). However, there were no difference in tenderness and free calcium concentrations between treatments for any other aging and retail display period.

There was a significant ($P < 0.01$) interaction between aging and treatments for lipid oxidation. Steaks from steers fed corn-only had higher TBARS values compared to steaks from steers fed WDGS ($P < 0.05$) at 21 d aging (Figure 3). This result contradicted the results from many other studies that showed feeding cattle high concentration of WDGS significantly increases lipid oxidation levels compared to feeding corn-only (Gill, 1996; Kinman et al., 2011; Senaratne et al., 2011).

No differences ($P > 0.1$) in sarcomere length between treatments were observed. The average sarcomere length of strip loins from WDGS and corn-only steers are 1.84 and 1.82 μm, respectively. Results of troponin-T degradation are shown in Table 3. An example of a Western blots used to quantify troponin-T degradation is shown in Figure 4. Western blot analyses indicated that there were no differences ($P > 0.10$) in troponin-T degradation between treatments in any of the aging or display period.

## DISCUSSION

When compared the beef SR membrane fatty acid profiles from this study to the beef muscle tissue fatty acid profiles (Domenech et al., 2014) from the same set of cattle, similar trends exist but they are extremely different in magnitude. In general,

concentrations of saturated fatty acids (SFA) and MUFA decreased, while PUFA quadrupled in beef SR membrane compared to the fatty acid profiles of beef muscle. The organelle, SR, is a sheet-like structure with more membrane surface than muscle and adipose cells. Our results show that more than half of the total lipids from the SR are phospholipids. Larick and Turner (1990) and Noci et al. (2005) reported that PUFA, especially 18:2, are predominately associated with the phospholipid fraction. Therefore, the higher content of PUFA in SR membrane than muscle tissue was expected.

The increase in 18:2 fatty acid and PUFA of the SR membrane from WDGS fed steers supported our hypothesis that feeding WDGS may impair SR membrane integrity. Modification of beef SR membrane fatty acid profiles by feeding WDGS likely occurred by the same mechanism that modification of beef muscle fatty acid profiles occurs. Although the majority of the unsaturated fatty acids (UFA) are fermented and biohydrogenated to SFA in the rumen by the microbes, WDGS has triple the amount of corn oil (Ham et al., 1994) compared to corn, which leads to greater deposition of 18:2 and PUFA in the muscle tissue and SR membrane. Furthermore, Cheah (1981) reported that free 18:2 fatty acid can stimulate the release of calcium and inhibit calcium uptake from the SR membrane. It is possible that 18:2 from the SR membrane released by endogenous phospholipase A2 induced the ryanodine receptor (RyR1) to release calcium, which contributed to the tenderness differences found in this study.

All individual MUFA and total MUFA decreased in SR membrane for WDGS fed steers. However, the reason for such a decrease is still unclear. Unlike 18:2, which is entirely derived from the diet, medium and long chain MUFA are products of biohydrogenation from dietary PUFA and SFA to MUFA conversion from delta-9-

desaturase (Wood et al., 2008; Smith et al., 2006). Perhaps, the corn-only steers needed to generate more MUFA from SFA because of less available PUFA in the plasma to incorporate in the organelle membrane, or the WDGS diet (high in PUFA) suppressed the stearoyl-CoA desaturase (SCD) gene, which is the gene that encodes for delta-9-desaturase (Chung et al., 2007).

Deaver Jr. et al. (1986), Brasitus et al. (1985) and Thi-Dinh et al. (1990) found that feeding rats a high unsaturated fat diet decreases the phospholipid concentration in liver, enterocytes and the plasma membrane of fat cells. Diets rich in PUFA, like pasture and WDGS, are known to increase the proportion of PUFA in muscle tissue phospholipids of beef (Dannenberger et al., 2006), fish (Huang et al., 1998) and pork (Nurnberg et al., 1998). Mead et al. (1980) further suggested lipid oxidation can increase the activity of phospholipases to remove esterified fatty acids. Perhaps, the higher PUFA content contributes to oxidation of SR membrane phospholipids, thus resulting in accelerated SR membrane phospholipid degradation and causing differences in phospholipid concentration between the two treatments. Ji and Takahashi (2006) reported that concentrations of SR membrane phospholipid are negatively correlated to sarcoplasmic calcium concentration during aging of pork and beef, and they hypothesized that calcium ions leaked into the sarcoplasm through channels formed by the degradation of phospholipids. It is possible that the decrease in SR membrane phospholipid concentration in WDGS samples contributes to the increase of free calcium and thereby improves tenderness.

Mlekusch et al. (1993) also found that feeding rats a diet high in PUFA increased concentration of PC, but decreased the concentration of PE in rat liver. However, no clear

explanation was given for the phenomenon. Beare and Kates (1964) showed that rat muscle tissue PC can incorporate 18:2 better than PE can. The present study showed that feeding WDGS increased 18:2 content of beef SR membrane by 35%. Such a shift of phospholipid profile found in Mlekusch et al. (1993) and this study can be explained by the increase of 18:2 content in feed. Phospholipid PE has the ability to enhance the activity of sarco-endoplasmic reticulum calcium ATPase (SERCA) in SR membrane through specific headgroup interactions (Hunter et al., 1999). It is possible that a reduction in SR membrane PE in WDGS-fed cattle impedes calcium influx, resulting in an increase of sarcoplasmic free calcium concentration.

Segers et al. (2011) showed that beef from cattle supplemented with 25 % dried distillers plus solubles (DDGS) for 100 d was more tender than beef from cattle supplemented with soybean meal at 7 d aging, while Depenbusch et al. (2009) reported sensory overall tenderness ratings increased linearly as dietary level of distillers grains increased from 0 to 75%. The tenderness and free calcium data from this study also agreed with the results from Senaratne (2012) and strongly supported our hypothesis. Extended aging beyond 2 d appeared to mitigate the tenderness and calcium efflux effects as there are fewer significant differences in these traits among the samples. Miller et al. (2011) also found differences in free calcium concentration between tender and tough samples early postmortem (24 and 48 h). However, the tenderness difference from that study was created through implants and a beta-agonist rather than diet. Unfortunately, they did not conduct any measurement on SR membrane integrity.

Lipid oxidation data from this study revealed another intriguing phenomenon, which beef from cattle fed corn-only was more oxidized than bef from cattle fed 50%

WDGS during retail display. However, this is not the first time that feeding distillers grains has been reported to control lipid oxidation. Song et al. (2013) also found that lipid oxidation values were not different between pigs fed 30% DDGS or corn-soybean based control diets. One explanation for such a phenomenon is that high concentration of sulfur in 50% WDGS diet (0.41% vs. 0.09% in corn only diet) may cause an antioxidant effect. The high concentration of sulfur may synthesize sulfur-containing amino acids and thus alleviate the oxidative stress induced by PUFA in WDGS (Song et al., 2013).

Sulfur-containing amino acids such as methionine, cystine, taurine, and glutathione have been studied extensively for their antioxidant properties (Atmaca, 2004; Battin and Brumaghim, 2009; Parcell, 2002). Hwang et al. (2000) fed 5% taurine to rats and observed decreased liver lipid oxidation from feeding diets containing 3% oxidized fish oil, which indicated that taurine may protect against lipid oxidation. Sulfur in water and feed exists in the form of sulfate; in ruminants, sulfate-reducing bacteria reduce sulfate to hydrogen sulfide, which is utilized to produce sulfur-containing amino acids (Drewnoski et al., 2014). It is possible that the increase in hydrogen sulfide also increased the production of sulfur-containing amino acids, thus triggered the antioxidant effect to protect PUFA in beef. Finally, measuring lipid oxidation on muscle tissue may not be the ideal way to measure SR membrane oxidation.

Oltra et al. (2008) also reported that feeding distillers grains has no effect on sarcomere length. Our hypothesis of beef tenderization is based on altered fatty acid profiles of the SR membrane. This effect might be a result of lipid oxidation of the SR membrane. There are still plenty of antioxidants available to prevent membrane lipid oxidation early postmortem, so the pre-rigor time course of calcium release should not be

affected by the higher PUFA content in the SR membrane. In fact, Stanley (1991) pointed out that the presence of PUFA in membranes can inhibit hydrocarbon chain packing thereby better preserving membrane integrity at lower temperature. Conversely, membrane lipid oxidation would occur sooner with more PUFA presented in the SR membrane.

Troponin-T degradation results indicated that the calpain activity was not different between treatments, but the WBSF results indicated otherwise. However, this is not the first time proteolysis data do not agree with WBSF results. Wheeler and Koohmaraie (1999) reported that troponin-T or desmin degradations in controlled and shortened longissimus and psoas major muscle with distinct tenderness differences were not different. Taylor et al. (1995) pointed out that the proteolytic degradation of specific proteins may be attributed to this poor relationship between WBSF and proteolysis. It is possible that proteolysis was different for some other proteins such as titin, desmin, vinculin, or nebulin in this study.

## Implications

In addition to lipid oxidation that might induce rapid calcium release found in this study, the contribution of protein oxidation should also be considered. The ryanodine receptor 1 (RyR1) of SR membrane possesses a number of highly reactive sulfhydryl (SH) groups that are susceptible to oxidation (Sun et al., 2001). It has been shown that oxidized SH groups can stimulate calcium release by forming disulfide bonds, which cause the RyR1 to maintain in the open state (Abramson and Salama, 1989). Hidalgo et al. (2000) further showed that oxidized RyR1 becomes active at low (μM) luminal

calcium concentrations and was not inhibited by high (m*M*) sarcoplasmic calcium concentrations. Although no measurement of SR membrane protein oxidation was made in this study, factors that promoted SR membrane lipid oxidation likely also promoted SR membrane protein oxidation. This study confirmed that feeding WDGS increased early postmortem tenderness and free calcium release, which likely was the result of increased total PUFA and decreased total phospholipids in the SR membrane from early onset of oxidation. Although the true mechanism and time course of membrane oxidation and free calcium release are still unclear, these results provide conceptual foundation for a new research perspective on meat tenderization.

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Table 1. Fatty acids profile of sarcoplasmic reticulum membrane from strip loins (*Longissimus lumborum*) from steers fed wet distillers grains plus solubles (WDGS) and corn-only finishing diets.

| | Dietary treatments | | | |
|---|---|---|---|---|
| Fatty Acids, % | 50% WDGS | Corn-only | SEM | *P*-value |
| C15:0 | 0.5 | 0.53 | 0.04 | 0.56 |
| C15:1 | 1.51 | 2.81 | 0.39 | 0.04 |
| C16:0 | 22.16 | 23.25 | 0.50 | 0.13 |
| C16:1 | 2.32 | 3.32 | 0.19 | < 0.01 |
| C17:0 | 0.95 | 0.94 | 0.08 | 0.94 |
| C17:1 | 0.97 | 1.19 | 0.04 | < 0.01 |
| C18:0 | 10.3 | 9.06 | 0.41 | 0.04 |
| C18:1 | 26.48 | 30.3 | 1.61 | 0.03 |
| C18:1V | 1.93 | 2.47 | 0.09 | < 0.01 |
| C18:2 | 16.81 | 12.46 | 1.32 | 0.03 |
| C18:3 | 0.42 | 0.39 | 0.05 | 0.63 |
| C20:3 | 1.3 | 1.39 | 0.12 | 0.59 |
| C20:4 | 4.97 | 5.57 | 0.47 | 0.37 |
| C20:5 | 0.48 | 0.52 | 0.08 | 0.71 |
| C22:4 | 0.8 | 0.85 | 0.11 | 0.75 |
| C22:5 | 0.22 | 0.19 | 0.02 | 0.08 |
| | | | | |
| SFA | 36.04 | 35.53 | 0.99 | 0.72 |
| UFA | 63.96 | 64.47 | 0.99 | 0.72 |
| SFA:UFA | 0.57 | 0.56 | 0.03 | 0.70 |
| MUFA | 33.09 | 38.52 | 1.28 | 0.01 |
| PUFA | 28.73 | 23.91 | 1.92 | 0.09 |

SFA = saturated fatty acids, UFA = unsaturated fatty acids, MUFA = monounsaturated fatty acids, and PUFA = polyunsaturated fatty acids

Table 2. Phospholipid and neutral lipid and total lipid profiles of sarcoplasmic reticulum membrane from strip loins (*Longissimus lumborum*) from steers fed wet distillers grains plus solubles (WDGS) and corn-only finishing diets.

| | Dietary treatments | | | |
|---|---|---|---|---|
| | 50% WDGS | Corn-only | SEM | *P*- value |
| Phospholipids, % | | | | |
| Phosphatidylcholine | 43 | 36.07 | 1.19 | < 0.01 |
| Phosphatidylethanolamine | 31.89 | 38.78 | 2.08 | 0.03 |
| Phosphatidylinositol | 2.86 | 2.66 | 0.24 | 0.56 |
| Phosphatidylserine | 1.03 | 1.15 | 0.14 | 0.53 |
| Sphingomyelin | 21.89 | 21.71 | 1.26 | 0.93 |
| Neutral Lipid, % | | | | |
| Mono, Di & Triacylglyceride | 91.03 | 88.11 | 1.81 | 0.12 |
| Cholesterol | 8.59 | 11.49 | 1.74 | 0.11 |
| Free Fatty Acids | 0.39 | 0.41 | 0.13 | 0.90 |
| Lipid, % | | | | |
| Phospholipid | 47.9 | 53.74 | 2.45 | 0.10 |
| Neutral Lipid | 52.1 | 46.26 | 2.45 | 0.10 |

Table 3. Troponin-T degradation of strip loins (*Longissimus lumborum*) from steers fed wet distillers grains plus solubles (WDGS) and corn-only finishing diets.

| | Degraded Troponin-T , % | | | | | |
|---|---|---|---|---|---|---|
| | 0 d retail display | | 7 d retail display | | | |
| Aging (d) | 50% WDGS | Corn-only | 50% WDGS | Corn-only | Pooled SEM | *P*-value |
| | | | | | 2.9 | 0.60 |
| 2 | 24.0 | 24.8 | 36.2 | 31.9 | | |
| 7 | 37.5 | 36.9 | 47.8 | 44.8 | | |
| 14 | 56.2 | 57.3 | 50.5 | 56.4 | | |
| 21 | 57.7 | 58.0 | 56.2 | 57.1 | | |

Figure 1. Warner-Bratzler shear force (WBSF) of strip loins (*Longissimus lumborum*) from steers fed wet distillers grains plus solubles (WDGS) and corn-only finishing diets with 0 d retail display.

Figure 2. Free calcium concentration of strip loins (*Longissimus lumborum*) aged for 2 d from steers fed wet distillers grains plus solubles (WDGS) and corn-only finishing diets.

Figure 3. Thiobarbituric acid assay values of strip loins (*Longissimus lumborum*) aged for 2, 7, 14, and 21 d from steers fed wet distillers grains plus solubles (WDGS) and corn only finishing diets.

Figure 4. Representative example (2 d aging) of Western blots that were used to quantify troponin-T degradation.

Figure 1.

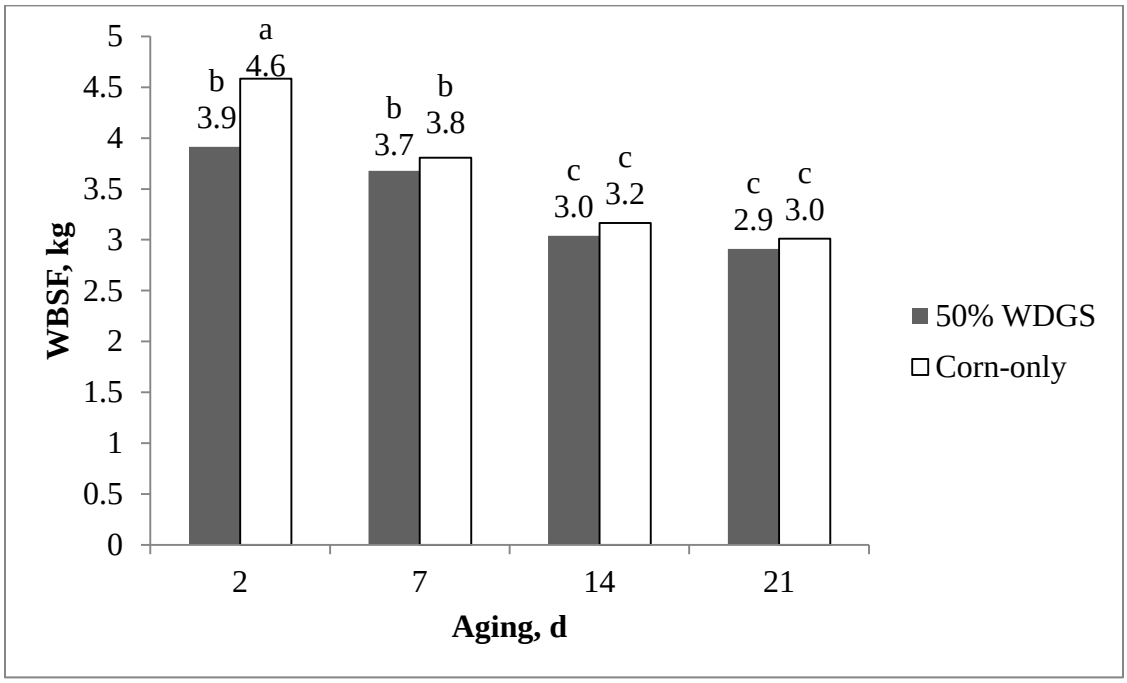

WBSF, kg
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
4.6
b
3.9
b
3.7
b
3.8
c
3.0
c
3.2
c
2.9
c
3.0
2
7
14
21
Aging, d
50% WDGS
Corn-only

Figure 2.

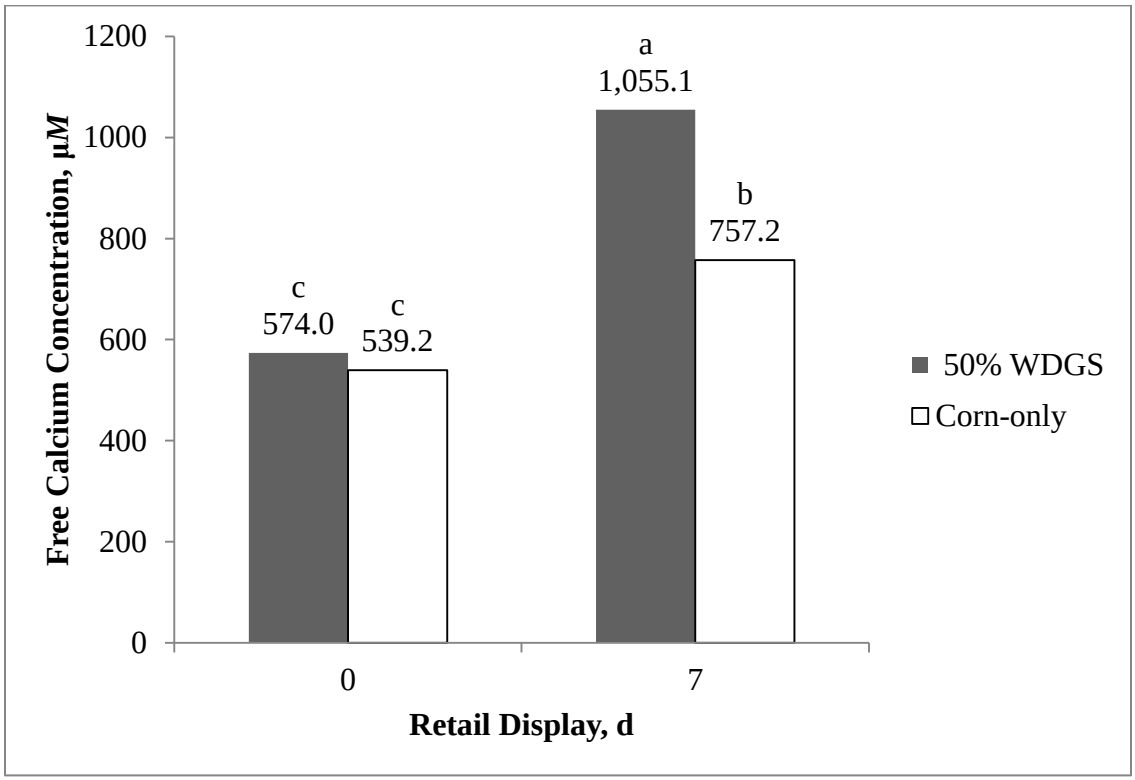

1200
1000
800
600
400
200
0
Free Calcium Concentration, µM
a
1,055.1
b
757.2
c
574.0
c
539.2
0
7
Retail Display, d
50% WDGS
Corn-only

Figure 3.

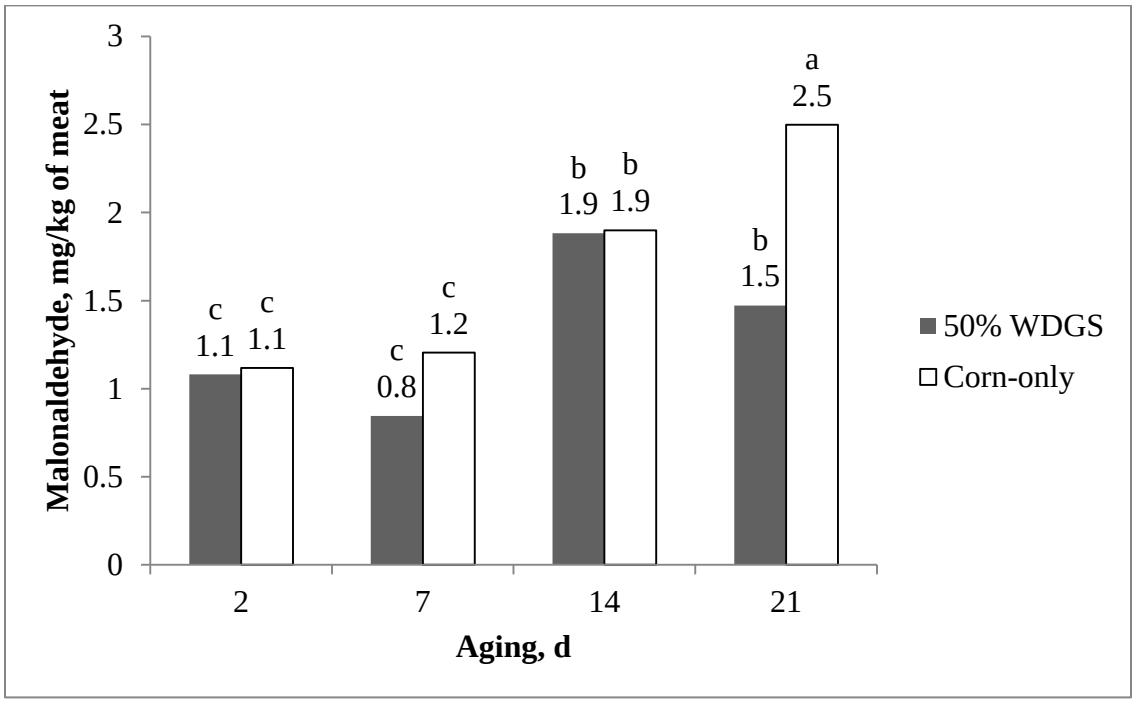
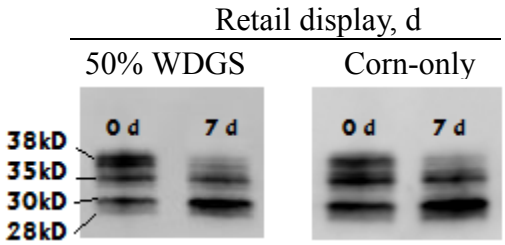

Malonaldehyde, mg/kg of meat
3
2.5
2
1.5
1
0.5
0
c 1.1
c 1.1
c 0.8
c 1.2
b 1.9
b 1.9
b 1.5
a 2.5
2
7
14
21
Aging, d
50% WDGS
Corn-only

Figure 4.


Retail display, d
50% WDGS
Corn-only
0 d
7 d
0 d
7 d
38kD
35kD
30kD
28kD

Running Head: Oxidation and antioxidants

# Effects of dietary antioxidant supplementation of cattle finished with 30% wet distillers grains plus solubles on fatty acid profiles and display life

**M. D. Chao, K. I. Domenech-Perez, H. R. Voegele, E. K. Kunze and C. R. Calkins[1]**

Department of Animal Science, University of Nebraska-Lincoln, Lincoln, NE 68583-0908

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[1]Corresponding author: Ccalkins1@unl.edu

**ABSTRACT**: Feeding wet distillers grains plus solubles (WDGS) in beef feedlot diets increases muscle tissue polyunsaturated fatty acids (PUFA) concentration and decreases beef display life, while antioxidants like vitamin E (E) and Agrado Plus (AG; Novus International) may mitigate such effects. Therefore, the objective of this study was to evaluate the effects of finishing diets containing WDGS and different antioxidants on muscle tissue fatty acid profiles, discoloration and lipid oxidation of retailed-displayed beef. One hundred and sixty cross-bred cattle were finished on either corn or 30% WDGS with 4 antioxidant treatments [E at 22.5 mg (50 IU)/hd/d– control; E at 450 mg (1,000 IU)/hd/d; AG at 3 g/hd/d (215 mg/kg of feed); a combination of 225 mg (500 IU)/hd/d of E and 3 g/hd/d (215 mg/kg of feed) of AG]. Ten Choice-grade strip loins from each treatment (n=80) were cut into steaks which were aged for 2, 7, and 14 d and placed under retail display conditions for 7 d. During the display period, steaks were evaluated daily for objective and subjective color scores. Lipid oxidation, muscle tissue fatty acid profile, and E and ethoxyquin concentrations were measured. Feeding WDGS decreased ($P < 0.05$) the proportions of 14:0, 14:1 and 16:1, but increased ($P < 0.05$) 15:0, 17:0, 17:1, 18:1 trans, 18:2, 20:1 and total PUFA in muscle when compared to the fatty acid profiles of cattle fed corn-only. In addition, steaks from the Corn+E treatment tended ($P < 0.10$) to have less 18:1 trans fatty acid when compared to the corn-only treatment. Supplementing E+AG or E alone was effective in reducing ($P < 0.01$) lipid oxidation for 0, 4 and 7 d of retail display in beef from cattle fed corn and was effective in maintaining color stability after 6 d of retail display ($P < 0.01$) in beef from both corn and WDGS diets. Supplementation of E or AG significantly increased ($P < 0.01$) muscle tissue E or ethoxyquin levels compared to the diets without E or AG supplementation. It was

interesting to note that WDGS+AG samples had higher ($P < 0.05$) muscle tissue E level compared to samples from Corn+AG. The inclusion of 30% WDGS altered fatty acid profiles of muscle tissue, but did not promote lipid and myoglobin oxidation compared to the corn-only diet. Feeding E alone was effective in reducing myoglobin and lipid oxidation in both diets, while supplementing AG showed a minor E-sparing effect when fed with E.

Key words: Beef, distillers grains, antioxidants, fatty acids, lipid oxidation, discoloration

## INTRODUCTION

In an effort to support energy independence, the U.S. has sought to promote production of ethanol as a source of biofuel. Distillers grains are the major by-product of ethanol production. Inclusion of 10 to 40% of distillers grains in finishing diets has shown to improve performance and carcass characteristics of feedlot cattle (Ham et al., 1994; Luebbe et al., 2012; Vander Pol et al., 2009). However, many studies (Koger et al., 2010; Mello et al., 2012b; Senaratne et al., 2011; Sherbeck et al., 1995) have also shown that meat products from cattle fed distillers grains are more readily oxidized during retail display, resulting in reduced shelf-life from discoloration and lipid oxidation. Feeding distillers grains to finishing cattle linearly increases polyunsaturated fatty acid (PUFA) levels in beef, and these negative effects on beef shelf-life are the direct results of reduced redox capacity from increased PUFA in muscle tissue (Mello et al., 2012b).

The most common method to improve color and oxidative stability of fresh meat is supplementation of dietary antioxidants. Vitamin E (E) has been shown to improve marketability of fresh meat products, maintain cellular integrity and delay lipid and

myoglobin oxidation effectively (decreased off-flavor and metmyoglobin formation) when used as a supplement in cattle (Maddock et al., 2003; Senaratne et al., 2011), pigs (Hoving-Bolink et al., 1998; Monahan et al., 1992; Pfalzgraf et al., 1995), and lambs (Santé-Lhoutellier et al., 2008). Agrado-Plus (AG; Novus International, Saint Charles, MO) also provides antioxidant activity, though results varied (Krumsiek and Owens, 1998; Walenciak et al., 1999). The synthetic antioxidant AG is a mixture of ethoxyquin (1,2-dihydro-6ethoxy-2,2,4-trimethylquinoline) and TBHQ (tertiary butylhydroquinone). Fat-soluble vitamins such as E are stabilized when AG is fed and may create an additive effect when AG is fed in combination with E (Lauridsen et al., 1995). Therefore, it is important to investigate the effects of dietary lipid and antioxidants on muscle tissue fatty acids and redox potential in order to further understand the overall changes of beef shelf-life resulting from diet modification. The objective of this study was to evaluate the effects of finishing diets containing wet distillers grains plus solubles (WDGS) and different antioxidants on muscle tissue fatty acid profiles, discoloration and lipid oxidation of retailed-displayed beef.

## MATERIALS AND METHODS

All animal use protocols were approved by the University of Nebraska-Lincoln's Animal Care and Use Committee.

### *Animals*

One hundred and sixty Continental X British cattle were blocked by BW, stratified by BW within each block, and assigned randomly to pens within block. Pens were randomly assigned to one of the eight treatments with two pens per treatment and

ten cattle per pen. Cattle were fed for 106 d on either a corn-only diet or a corn based diet with 30% WDGS (DM basis) with four antioxidant treatments. The eight treatments were 1) a corn-only diet with E supplementation at 22.5mg (50 IU)/hd/d (control); 2) a corn-only diet with E supplementation at 450 mg (1,000 IU)/hd/d (Corn+E); 3) a corn-only diet with AG supplementation at 3 g/hd/d (215 mg/kg of feed) (Corn+AG); 4) a corn-only diet with a combination of 225 mg (500 IU)/hd/d of E and 3 g/hd/d (215 mg/kg of feed) of AG (Corn +E+AG); 5) a corn based diet with 30% WDGS with E supplementation at 22.5 mg (50 IU)/hd/d (WDGS); 6) a corn based diet with 30% WDGS with E supplementation at 450 mg (1,000 IU)/hd/d (WDGS+E); 7) a corn based diet with 30% WDGS with AG supplementation at 3 g/hd/d (215 mg/kg of feed) (WDGS+AG); 8) a corn based diet with 30% WDGS with a combination of 225 mg (500 IU)/hd/d of E and 3 g/hd/d (215 mg/kg of feed) of AG (WDGS+E+AG).

*Sample Collection, Fabrication and Preparation*

All cattle were harvested at a commercial abattoir. After 48 h of postmortem chilling, 80 carcasses were selected (10 carcasses from each treatment). Carcass selection was based on treatment and quality grade (Choice). The strip loins were collected, vacuumed-packaged and transported to Loeffel meat laboratory at the University of Nebraska-Lincoln.

Strip loins from the left sides of the carcasses were fabricated on d 2 and 7 of postmortem aging, and strip loins from the right sides of carcasses were fabricated on d 14 of postmortem aging. Each strip loin was fabricated into 1 color sample (2.54 cm) and 2 lab samples (1.27 cm) for each aging period from the anterior to the posterior end of the

loin muscle. The second lab sample was further divided in equally-sized halves for retail display. The remainders of the strip loins were immediately vacuum packaged (3 mm STD barrier, Prime Sources, St. Louis, OM) on a Multivac Packaging machine (MULTIVAC C500, Multivac Inc., Kansas city, MO) and aged to the next designated aging period.

Upon fabrication, steaks were overwrapped with oxygen permeable polyvinyl chloride film (PSM18, Prime Source, St. Louis, MO) and subjected to retail display conditions (2 ± 2$^{o}$C, and exposed to continuous 1,000-1,800 lux warm white fluorescence lighting). For each aging period, samples for lipid oxidation assessments were obtained on d 0, 4 and 7 of retail display, and the color sample was used to evaluate daily objective color and subjective discoloration scores during the 7 d retail display period. Fatty acid profiles, E and ethoxyquin samples were obtained on d 0 of retail display after 14 d of aging. At the end of the allotted treatments, all samples were vacuum packaged and frozen at -80$^{o}$C until analyzed. The lab samples were pulverized in liquid nitrogen with a blender (model 51BL32, Waring Commercial, Torring, CT).

*Discoloration*

A five-person trained panel consisting of graduate students in the Animal Science Department at the University of Nebraska-Lincoln subjectively evaluated discoloration of each steak as a percentage (0 – 100%) of total surface area. Panelists were trained using a system of open discussion and a range of discoloration.

*Objective color*

Objective color measurements were obtained for CIE L*, a*, and b* values using a Minolta CR-400 colorimeter (Minolta, Osaka, Japan) set at a D65 light source and 2° observer with an 8 mm diameter measurement area. The colorimeter was calibrated daily using a white ceramic tile provided by the manufacturer, and color measures were obtained at 0,1, 2, 3, 4, 5, 6 and 7 d of display by averaging 6 reading from different areas of the steak surface. The CIE a* measurements, a measure of red to green, were used to determine color stability of the steaks.

*Fatty Acid Profiles*

Total lipid was extracted following the procedure described by Folch et al. (1957). After extraction, lipids were converted to fatty acid methyl esters according to Morrison and Smith (1964) and Metcalfe et al. (1966). The samples were analyzed using gas chromatography (Hewlett-Packard 6890 FID GC System; Agilent Technologies, Santa Clara, CA) with setups described by Mello et al. (2012b).

*Lipid Oxidation*

Samples were prepared according to the thiobarbituric acid assay (TBA) described by Ahn et al. (1998). Duplicate 200 μL of supernatant from each sample was transferred to 96-well plates and read with a microplate spectrophotometer (Model Epoch, Biotek, Winooski, VT) at 540 nm. All 96-well plates had standards to calculate standard curves, and each sample was calculated as mg of malonaldehyde per kg of tissue using the standard curve from each plate.

*Vitamin E and Ethoxyquin Concentrations*

The E level in muscle tissue was quantified by the procedure described by Nierenberg and Nann (1992) with modifications. Briefly, 1 gram of powdered meat sample was combined with 3 mL of 6% pyrogallol (to minimize oxidation) and 1 mL of saturated potassium hydroxide. The mixture was digested for 30 min at 70$^{o}$ C, and 5 mL of deionized distilled water (dd$H_2O$) and 1 mL of hexane containing 0.05% butylated hydroxytoluene (BHT) was added after cooling. The mixture was centrifuged at 10,000 x *g* for 2 min, and the supernatant was collected. The extraction process was repeated for 3 times. Pooled supernatants were evaporated to dryness under nitrogen flush, and the residue was resuspended in 200 µL of tetrahydrofuran. The sample was brought to 300 µL with mobile phase. The mobile phase consisted of acetonitrile: tetrahydrofuran: methanol: 1% ammonium acetate in dd$H_2O$ (65:25:6:4, v/v/v/v) containing 0.1% BHT and 0.05% trimethylamine. Fifty µl of samples were injected into an HPLC system (Waters Associates instruments; 600E solvent delivery system and 2487 UV detector, Milford, MA) with a reversed phase Microsorb-MV100 C18 column (5 µm, 250 × 4.6 mm; Rainin Instruments, Woburn, MA). The flow rate was set at 1.0 mL/min and absorbance was read at 292 nm for E.

Ethoxyquin in muscle tissue was extracted by the method b extraction procedure described by Aoki et al. (2010). Briefly, 5 grams of powdered meat samples were homogenized with 20 ml of methanol and 10 g of sodium sulfate anhydride. The homogenate was centrifuged at 4,000 x *g* for 5 min, and the supernatant was collected. The extraction procedure was repeated one more time, and the supernatant was combined. Ten milliliters of one-propanol was added to the extraction solution to prevent

bumping and the solution was evaporated to dryness. The residue was dissolved in 5 mL of acetonitrile: water (2:3, v/v), sonicated, and filtered through a 0.45 μm membrane filter (Rainin Instruments). Five μl filtered sample solution was injected into HPLC system (Waters Associates instruments; 600E solvent delivery system, and 474 florescence detector). The mobile phase consisted of acetonitrile: 0.01M ammonium acetate (4:1 v/v). The flow rate was set at 0.2 mL/min, and the excitation and emission wavelengths for fluorescence monitoring of ethoxyquin were 360 and 436 nm, respectively.

*Statistical Analysis*

Data for TBA were analyzed as a split-split plot design with dietary treatments as the whole-plot, aging period as the split-plot and retail display time as the split-split plot. Color data were analyzed as a split-split-plot repeated measures design with dietary treatments as the whole-plot, aging period as the sub-plot and retail display d as the repeated measures. The E and ethoxyquin concentrations and fatty acid profiles were analyzed as a completely randomized design. Data were analyzed using the GLIMMIX procedure of SAS (version 9.2, Cary, NC, 2009). Separation of means was conducted using LSMEANS procedure with PDIFF or SLICEDIFF options at $P < 0.05$. In addition, the CONTRAST statements in SAS were used to compare effects of feeding Corn vs. WDGS, E vs. no E, AG vs. no AG and E+AG vs. no E+AG.

## RESULTS

Fatty acid profiles are shown in Table 1. Fatty acid profiles revealed some differences among steaks from cattle fed different diets. In general, muscle tissue from cattle fed WDGS had higher ($P < 0.01$) concentrations of pentadecanoic acid (15:0),

heptadecanoic (17:0), heptadecenoic acid (17:1), elaidic acid (18:1 trans), linoleic acid (18:2), eicosenoic acid (20:1), and total PUFA, but lower ($P < 0.01$) concentrations of myristic (14:0), myristoleic acid (14:1), palmitoleic acid (16:1), and tended ($P < 0.10$) to have lower concentrations of linolenic acid (18:3) and docosapentaenoic acid (22:5) compared to muscle tissue from cattle fed corn only. Furthermore, the Corn+E treatment tended ($P < 0.10$) to have less 18:1 trans fatty acid compared to the control.

With d 7 of retail display, LS means of Minolta CIE a* are separated and ranked in Table 2, and graphical presentation is depicted in Figure 1. There was a significant interaction between retail display d and treatments for a* value ($P < 0.01$). There were no differences among steaks of different treatments in a* value from d 0 to 4 (data not shown). On d 5 of the retail display, steaks from WDGS+E+AG treatment had significantly higher ($P < 0.05$) a* values than the steaks from rest of the treatments. Although only steaks from Corn+E+AG and WDGS+E+AG treatments had significantly higher ($P < 0.05$) a* values compared to the steaks without E treatments, the trend that separated out the ones with E treatments and the ones without E treatments could clearly be seen here on d 6 of the retail display. On d 7 of the retail display, all steaks from cattle supplemented with E or the combination of E and AG had significantly higher ($P < 0.05$) a* values than the steaks without E treatments with one exception. On d 6 of the retail display, a* values of steaks from WDGS+AG treatments were not different from a* values from Corn+E+AG treated steaks and stayed close together with other E-treated steaks. On d 7 of the retail display, it became clear that a* values from WDGS+AG treatments were significantly higher ($P < 0.05$) compared to the a* values from the rest of

the steaks without E treatment. The Minolta CIE L* and b* values were not different among treatments in any of the display period.

With d 7 of retail display, LS means of discoloration are separated and ranked in Table 2, and graphical presentation is depicted in Figure 1. There was a significant interaction between retail display d and treatments for discoloration scores ($P < 0.01$). There were no differences among treatments in discoloration scores from d 0 to 5 (data from d 0 to 4 not shown). Similar to a* values, although only steaks from control and Corn+AG treatments had significantly higher ($P < 0.05$) discoloration than the steaks with E treatments, the trend that separated out the ones with E treatments and the ones without E treatments could clearly be seen here on d 6 of the retail display. At d 7 of the retail display period, all steaks from cattle supplemented with E or the combination of E and AG were significantly less discolored ($P < 0.01$) than steaks from cattle not supplemented with E. It is also important to note that the discoloration was still minimal at d 7 of retail display, and the divergence in discoloration would be expected to continue if the display period progressed.

Lipid oxidation data are presented in Table 3. There was a significant interaction between retail display d and treatments for lipid oxidation ($P < 0.01$). Lipid oxidation values followed a similar pattern with the subjective color scores numerically, but there were no statistical differences in lipid oxidation among cattle fed a 30% WDGS diets supplemented with antioxidants or not. A reduction in oxidation rates due to E and/or AG supplementation was observed at all three retail display period only when cattle were fed the corn-only diets ($P < 0.01$). The E+AG supplementation was most effective, followed by E only, and then the AG only supplementation. Comparing diet treatment without any

antioxidant supplementation, steaks from the control cattle had higher lipid oxidation values compared to steaks from cattle fed 30% WDGS ($P < 0.01$). There was one final interesting observation. The WDGS+AG combination yielded a significantly lower ($P < 0.05$) lipid oxidation value than the Corn+AG combination after d 4 and 7 of retail display.

Diets with E supplementation had significantly more ($P < 0.01$) muscle tissue E levels compared to the diets without E supplementation (Table 2). Again, it is interesting to note that WDGS+AG samples had higher ($P < 0.05$) muscle tissue E level compared to samples from Corn+AG. Diets with AG (ethoxyquin and TBHQ) supplementation also significantly increased muscle tissue ethoxyquin levels compared to diets without AG supplementation (Table 3).

## DISCUSSION

The diet effect on fatty acid profiles matches the results from many previous studies, and the detailed mechanism of WDGS influenced PUFA deposition in beef has been thoroughly discussed many times (Buttrey et al., 2013; Domenech et al., 2014a; Gill et al., 2008; Mello et al., 2012b). Briefly, although the majority of the unsaturated fatty acids (UFA) are fermented and biohydrogenated to saturated fatty acids (SFA) in the rumen, WDGS has double the amount of PUFA (Ham et al., 1994) compared to corn, which leads to greater bypass of PUFA to the duodenum (Vander Pol et al., 2009), thus increased the deposition of PUFA in the muscle (Mello et al., 2012b).

Slight shifts of a few long and short chain SFA and MUFA were seen in this study, and these results match the outcomes reported by Mello et al. (2012a) from feeding

different levels of modified distillers grains plus solubles in beef muscle tissues. Ruminant trans fatty acids and long chain SFA and MUFA are mainly synthesized via biohydrogenation from microbial fermentation of PUFA (Lock and Bauman, 2004). Therefore, the increased in long chain MUFA and trans fatty acids from WDGS treatments in this study are likely the direct effect of ruminal biohydrogenation from increased PUFA of the WDGS diets. Conversely, short and medium chain SFA and MUFA can be generated through de novo fatty acid synthesis in animals (Wood et al., 2008). The increase in short and medium chain SFA and MUFA from the corn treatments is likely synthesized in the cattle to compensate for the lower concentration of dietary fat in the corn-only diets. There is no doubt that the responses of individual fatty acids to dietary treatments in the current study are the results of a combination of diet and enzymatic function.

In most studies, muscle tissue fatty acid composition was not affected by dietary E supplementation in beef (Mello, 2010) and lamb (Álvarez et al., 2008; Kasapidou et al., 2009). However, results from this study showed a tendency for E supplementation to inhibit the production of 18:1 trans fatty acid in the corn diet. Juarez et al. (2010) also found that E supplementation to a barley-based concentrate diet decreased the production of 18:1 trans fatty acid. Furthermore, Pottier et al. (2006) reported the same shift in milk composition when cows were supplemented with a high concentration of E. Both authors speculated that E could either influence ruminal pathways of PUFA biohydrogenation, acting either as an inhibitor of 18:1 trans-producing bacteria or as an electron donor to provide the electrons during the isomerization of the 18:2. Unfortunately, none of these hypotheses has been confirmed yet. It is important to note that this decrease in 18:1 trans

trend is only observed in Corn+E treatment, but not in WDGS+E treatment. It is possible that the strong redox potential found in beef from WDGS fed cattle in this study mitigated the antioxidant effect of E. The 18:1 trans fatty acids have been linked to cardiovascular health risks in human and animal models (Bauchart et al., 2007; Hodgson et al., 1996). Supplementation of E to cattle may provide human health benefits on top of the well-known shelf-life improvement effect, which may be another incentive for the producers to widely apply such practice.

In muscle, myoglobin is responsible for the bright cherry red color. When myoglobin is oxidized to metmyoglobin, discoloration starts to occur (Liu et al., 1995). This study did not show that feeding 30% WDGS significantly decreased color stability (for both objective and subjective color scores) compared to a corn-only diet as many other studies have (Kinman et al., 2011; Mello et al., 2012b; Senaratne, 2012; Senaratne et al., 2011). Color stability of beef from cattle supplemented with WDGS is usually reduced due to the propagation of oxidation caused by high concentration of PUFA in WDGS (Kinman et al., 2011). Even with the distinct difference in PUFA content, steaks from both dietary treatments in this study exhibited very good color stability. Roeber et al. (2005) reported that finishing diets including high levels (40-50%) of distillers grains may negatively impact color stability of strip loin steaks, but low to moderate levels of WDGS (10%-25%) could be included in the diet with no detrimental effects. Perhaps, 30% WDGS in the finishing diet might simply be insufficient to drive the myoglobin oxidation process and distinguish WDGS from corn.

The E supplementation was effective in this study to maintain both a* values and discoloration scores in steaks from cattle fed either corn or WDGS diets, and this result is

in agreement with many others on the efficacy of E to maintain color stability (Faustman et al., 1989; Maddock et al., 2003; Senaratne et al., 2011). The detailed mechanism of E's role in preventing lipid and myoglobin oxidation has been discussed in a thorough review by Schaefer (2007). Briefly, E is a membrane-bound antioxidant that reduces a neighboring free radical by donating an electron from the hydroxyl group on carbon 6 of the chromanol ring (Liu et al., 1995). Due to its advantageous location in the membrane, E is the most effective product to improve shelf-life of fresh beef products (Schaefer, 2007).

In this research, WDGS+AG treatment improved a* values when compared to WDGS-only treatment. This result disagreed with Senaratne's (2012) finding that reduction in steak discoloration rates due to AG supplementation was only observed when cattle were on corn-only diets. Senaratne (2012) suggested that the decreased redox potential in beef from cattle fed WDGS overwhelms the antioxidant benefit of AG, while it is the exact opposite in this study. The higher oxidation status of beef from corn-only cattle in this study might overwhelm the small fraction of ethoxyquin that was incorporated into the muscle tissue. The main purpose of synthetic antioxidants like ethoxyquin and TBHQ is for protection of the feed ingredients against oxidative degradation during storage and processing (McCarthy et al., 2001; Tavárez et al., 2011). Therefore, an additive effect is expected through preservation of lipid-soluble vitamins like E both in feed and in animal tissues (Lauridsen et al., 1995). In this study, the E+AG combinations for both diets were numerically the least discolored and had the best a* values when compared to other treatments even though both E+AG treatments contained less E than the E only treatment. This clearly demonstrated synergistic effect for E and

AG. However, in a study that evaluated the effect of ethoxyquin and E on beef shelf-life, Maddock et al. (2003) concluded that no synergistic effects of ethoxyquin and E were observed. The water-soluble TBHQ in AG may play a role in the synergistic effects observed in this study since Maddock et al. (2003) used ethoxyquin only instead of AG as a treatment for their study.

This study also found that beef from cattle fed corn-only was more oxidized than beef from cattle fed WDGS in all display periods, which is in contrast to the results from many others (Gill, 1996; Kinman et al., 2011; Senaratne et al., 2011). However, Domenech et al. (2014b) reported that beef from cattle fed a corn-only diet had the highest lipid oxidation value when compared to diets with different levels of full or de-oiled WDGS, while Song et al. (2013) found that lipid oxidation values were not different in pigs fed 30% dried distillers grains plus solubles (DDGS) or corn-soybean based control diets. A thorough study evaluating different levels of DDGS on the lipid oxidation and antioxidant status of chicken breast revealed that inclusion of DDGS up to 25 % in broiler diet had no effect on lipid oxidation nor total antioxidant capacity of chicken breast (Min et al., 2012). Song et al. (2013) suggested that feeding distillers grains may cause a vitamin E-sparing effect by synthesizing sulfur-containing antioxidant peptides and thus alleviating the oxidative stress induced by oxidized lipids in DDGS. Perhaps, WDGS induced sulfur-containing antioxidant formation, thus alter the results of this study.

Supplementation of E has again proven its efficacy to reduce lipid oxidation when cattle were fed a corn-only diet in this study. The ability for E to inhibit lipid oxidation works through the same way as its ability to reduce myoglobin oxidation (Schaefer,

2007), and has been discussed in many other studies (Faustman et al., 1989; Maddock et al., 2003; Senaratne et al., 2011). The results from this study also agreed with Krumsiek and Owens (1998) and Senaratne (2012) that feeding AG to cattle fed corn-only improved redox potential during retail display. A synergistic effect of E and AG was observed numerically, but not statistically, for lipid oxidation in this study. The effect of E, AG or the combination of E and AG in reducing lipid oxidation for beef steaks from WDGS treatment is likely diminished because of the already low level of lipid oxidation.

Arnold et al. (1993) reported that discoloration, lipid oxidation and E concentrations in muscle tissues are closely correlated, and E accumulation in muscle tissue is known to be influenced by dose and duration of feeding (Schaefer, 2007). In this study, the accumulation of E in muscle tissue was directly influenced by dosage except for WDGS+AG treatment. Han et al. (1999) reported that supplementation of AG to transport-stressed heifers increased blood E levels during the first 68 d of their arrival, compared to the control. On top of preserving E stability in feed, Lauridsen et al. (1994) further suggested that the small amount of ethoxyquin preserved in animal tissues might continue to protect α-tocopherol from oxidative deterioration. Based on the literature, it is clear that feeding AG or ethoxyquin can preserve dietary E to create a sparing effect (all cattle used in this study were supplemented with at least 50 IU of E). However, it is unclear why such an effect was only observed in WDGS-supplemented cattle in this study. Choat et al. (2002) found that AG supplementation to a corn-only diet had no effect on serum E compared with controls during last 25 d of feeding of feedlot steers. Most of these sparing effects were observed in E deficient or immunologically stressed animals; perhaps, PUFA from WDGS supplementation utilized much of the cattle's antioxidant

capacity of plasma E, thus created a situation with lower levels of plasma E which allowed for the sparing effect to be observed.

Finally, this study confirmed that the level of ethoxyquin accretion in muscle tissues has a direct effect on lipid and color stability. Although most ethoxyquin is broken down in the body and excreted in the urine, small amounts of ethoxyquin can be detected in liver, kidney, adipose and muscle tissue of rats fed ethoxyquin (Burka et al., 1996). Bohne et al. (2008) also reported that ethoxyquin and ethoxyquin metabolites could be seen in the muscles of Atlantic salmon after feeding ethoxyquin-supplemented fish meal. The Corn+AG treatment exhibited a reduction in lipid oxidation when compared to the control, but had low tissue E level. The most logical explanation for such an antioxidant effect is the residual ethoxyquin detected in the muscle tissue.

## CONCLUSION

In conclusion, the inclusion of 30% WDGS increased PUFA content in muscle tissue, but did not promote lipid and myoglobin oxidation compared to the corn-only diet. Myoglobin oxidation was effectively suppressed by the combination of E+AG or E supplementation alone for both diets, while lipid oxidation was suppressed by E and E+AG combination in the corn-only diet. The AG supplementation alone only exhibited minor antioxidant effect for display life. It is intriguing to repeatedly find samples with more PUFA to exhibit better redox potential than samples with less PUFA, and this phenomenon warrant further investigation as a mean to truly understand the effect of distillers grains on beef display life.

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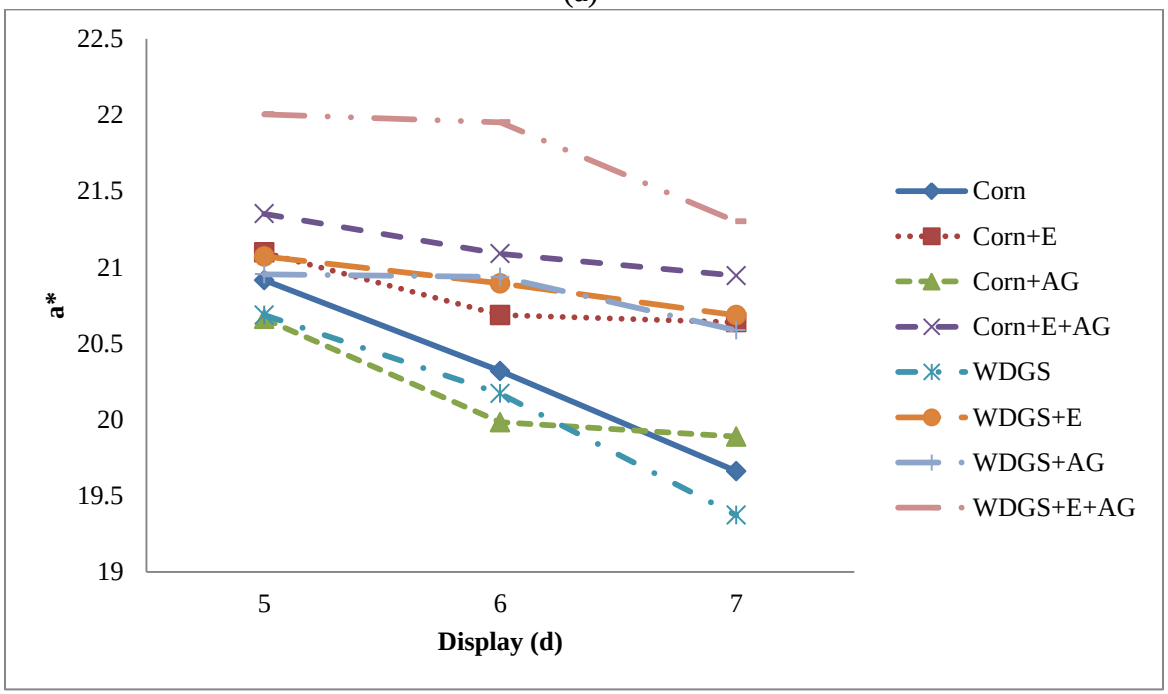
Figure 1. a) Objective redness (a*) and b) discoloration of strip steaks (*m. longissimus lumborum*) from cattle fed corn only or wet distillers grains plus solubles (WDGS) supplemented with or without vitamin E (E) or Agrado (AG) or a combination of E and AG from d 5 to 7 under retail display conditions.

Figure 1.

(a)


22.5
22
21.5
21
20.5
20
19.5
19
a*
5
6
7
Display (d)
Corn
Corn+E
Corn+AG
Corn+E+AG
WDGS
WDGS+E
WDGS+AG
WDGS+E+AG

(b)

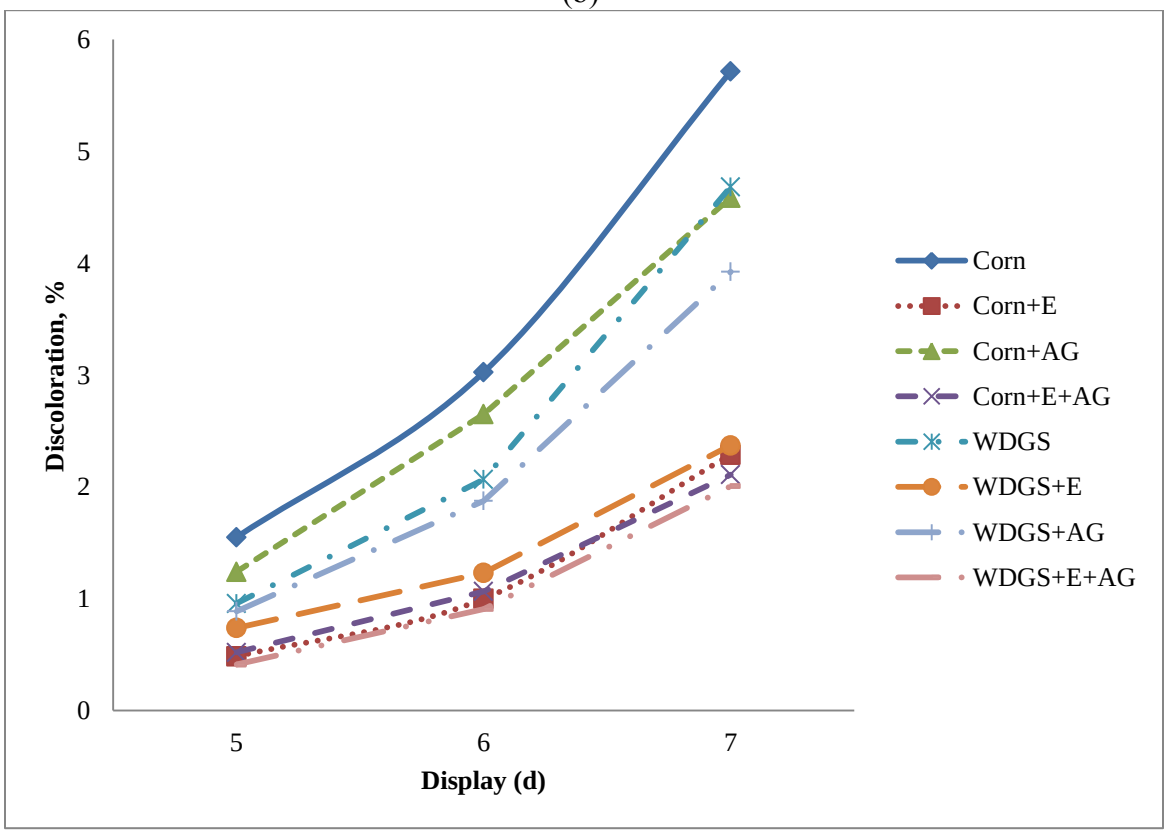

6
5
4
3
2
1
0
Discoloration, %
5
6
7
Display (d)
Corn
Corn+E
Corn+AG
Corn+E+AG
WDGS
WDGS+E
WDGS+AG
WDGS+E+AG

Table 1. Fatty acids profile (%) of strip steaks (*m. longissimus lumborum*) from steers fed corn only or wet distillers grains plus solubles (WDGS) supplemented with or without vitamin E (E) or Agrado (AG) or a combination of E and AG.

| | Dietary treatments | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Fatty Acids | Corn | Corn+E | Corn+AG | Corn+E+AG | WDGS | WDGS+E | WDGS+AG | WDGS+E+AG | SEM | *P*-value |
| C14:0 | 2.00$^{abc}$ | 2.34$^{ab}$ | 2.23$^{abc}$ | 2.39$^{a}$ | 1.83$^{bc}$ | 1.73$^{c}$ | 2.01$^{abc}$ | 1.73$^{c}$ | 0.12 | <0.01 |
| C14:1 | 0.61$^{abc}$ | 0.66$^{ab}$ | 0.60$^{abc}$ | 0.68$^{a}$ | 0.45$^{bc}$ | 0.42$^{c}$ | 0.60$^{abc}$ | 0.51$^{abc}$ | 0.05 | <0.01 |
| C15:0 | 0.42$^{bc}$ | 0.37$^{c}$ | 0.42$^{bc}$ | 0.47$^{abc}$ | 0.60$^{a}$ | 0.52$^{abc}$ | 0.59$^{a}$ | 0.53$^{ab}$ | 0.03 | <0.01 |
| C15:1 | 0.67 | 0.63 | 0.56 | 0.62 | 0.52 | 0.52 | 0.53 | 0.59 | 0.05 | 0.25 |
| C16:0 | 24.73 | 23.34 | 22.34 | 23.43 | 21.64 | 22.24 | 20.98 | 22.84 | 1.28 | 0.54 |
| C16:1 | 2.17$^{ab}$ | 2.43$^{ab}$ | 2.69$^{a}$ | 2.73$^{a}$ | 1.91$^{b}$ | 2.01$^{b}$ | 2.25$^{ab}$ | 1.88$^{b}$ | 0.21 | 0.02 |
| C17:0 | 1.48$^{cd}$ | 1.27$^{d}$ | 1.42$^{cd}$ | 1.65$^{abc}$ | 2.43$^{a}$ | 2.14$^{abc}$ | 1.93$^{abcd}$ | 2.28$^{ab}$ | 0.17 | <0.01 |
| C17:1 | 1.01$^{ab}$ | 0.90$^{b}$ | 1.08$^{ab}$ | 1.16$^{ab}$ | 1.48$^{a}$ | 1.35$^{ab}$ | 1.51$^{a}$ | 1.33$^{ab}$ | 0.12 | <0.01 |
| C18:0 | 14.95 | 13.82 | 13.15 | 14.25 | 15.33 | 16.76 | 13.12 | 15.19 | 1.19 | 0.37 |
| C18:1T | 2.02$^{dc}$ | 1.44$^{d}$ | 2.05$^{bcd}$ | 1.85$^{d}$ | 3.50$^{a}$ | 3.25$^{a}$ | 3.03$^{abc}$ | 3.15$^{ab}$ | 0.24 | <0.01 |
| C18:1 | 32.36 | 34.52 | 36.23 | 34.17 | 33.14 | 34.29 | 34.63 | 31.47 | 2.12 | 0.84 |
| C18:1V | 1.07 | 1.22 | 1.44 | 1.27 | 1.09 | 1.04 | 1.25 | 1.16 | 0.12 | 0.19 |
| C18:2 | 3.58$^{bc}$ | 3.09$^{c}$ | 3.39$^{c}$ | 3.60$^{bc}$ | 5.48$^{a}$ | 4.77$^{ab}$ | 4.87$^{ab}$ | 4.88$^{ab}$ | 0.31 | <0.01 |
| C18:3 | 0.21 | 0.19 | 0.2 | 0.2 | 0.25 | 0.28 | 0.21 | 0.25 | 0.02 | 0.1 |
| C20:1 | 0.49$^{ab}$ | 0.41$^{ab}$ | 0.43$^{ab}$ | 0.38$^{b}$ | 0.54$^{ab}$ | 0.59$^{a}$ | 0.64$^{a}$ | 0.50$^{ab}$ | 0.05 | <0.01 |
| C20:3 | 0.27 | 0.25 | 0.26 | 0.28 | 0.24 | 0.24 | 0.23 | 0.27 | 0.03 | 0.63 |
| C20:4 | 0.75 | 0.73 | 0.71 | 0.8 | 0.63 | 0.66 | 0.71 | 0.73 | 0.06 | 0.66 |
| C22:5 | 0.29 | 0.22 | 0.2 | 0.21 | 0.17 | 0.17 | 0.18 | 0.22 | 0.03 | 0.06 |
| SFA[1] | 43.5 | 41.06 | 39.55 | 42.19 | 39.17 | 43.38 | 38.51 | 42.51 | 2.15 | 0.57 |
| MUFA[1] | 41.38 | 41.86 | 45.15 | 43.12 | 45.51 | 42.98 | 43.93 | 39.22 | 2.5 | 0.71 |
| PUFA[1] | 4.60$^{b}$ | 4.09$^{b}$ | 4.65$^{b}$ | 4.92$^{ab}$ | 6.58$^{a}$ | 5.68$^{ab}$ | 5.89$^{ab}$ | 5.85$^{ab}$ | 0.42 | <0.01 |

[1]SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, and PUFA = polyunsaturated fatty acids

[a-d] Within a row, means without a common superscript differ at $P \leq 0.05$.

Table 2. Ranking of objective redness (a*) and discoloration (%) of strip steaks (*m. longissimus lumborum*) from steers fed corn only or wet distillers grains plus solubles (WDGS) supplemented with or without vitamin E (E) or Agrado (AG) or a combination of E and AG after 7 d of retail display.

| Dietary treatments | a* | Dietary treatments | Discoloration |
|---|---|---|---|
| WDGS+E+AG | 21.30[a] | WDGS+E+AG | 2.01[b] |
| Corn+E+AG | 20.94[a] | Corn+E+AG | 2.11[b] |
| WDGS+E | 20.69[ab] | Corn+E | 2.29[b] |
| Corn+E | 20.69[ab] | WDGS+E | 2.37[b] |
| WDGS+AG | 20.59[ab] | WDGS+AG | 3.92[c] |
| Corn+AG | 19.89[bc] | Corn+AG | 4.59[ac] |
| Corn | 19.66[c] | WDGS | 4.68[ac] |
| WDGS | 19.37[c] | Corn | 5.71[a] |
| SEM | 0.31 | | 0.57 |
| *P*-value | < 0.01 | | < 0.01 |

[a-c] Within a column, means without a common superscript differ at $P \leq 0.05$.

Table 3. Lipid oxidation value (TBA; malonaldehyde mg/kg of meat), vitamin E (E; ug/g) and ethoxyquin (ug/100 g) concentrations of strip steaks (*m. longissimus lumborum*) from steers fed corn only or wet distillers grains plus solubles (WDGS) supplemented with or without E or AG or a combination of E and AG.

| | | Dietary treatments | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | d | Corn | Corn+E | Corn+AG | Corn+E+AG | WDGS | WDGS+E | WDGS+AG | WDGS+E+AG | SEM | *P*-value |
| TBA | | | | | | | | | | 0.32 | < 0.01 |
| | 0 | 1.83$^{a}$ | 1.22$^{ab}$ | 1.17$^{ab}$ | 0.99$^{b}$ | 0.88$^{b}$ | 1.19$^{ab}$ | 1.19$^{ab}$ | 1.11$^{ab}$ | | |
| | 4 | 3.51$^{a}$ | 1.97$^{bc}$ | 2.36$^{c}$ | 1.36$^{b}$ | 1.73$^{bc}$ | 1.66$^{bc}$ | 1.82$^{bc}$ | 1.50$^{b}$ | | |
| | 7 | 5.04$^{a}$ | 2.45$^{b}$ | 3.71$^{c}$ | 1.65$^{b}$ | 2.99$^{bc}$ | 2.17$^{b}$ | 2.78$^{b}$ | 1.82$^{b}$ | | |
| E | | 2.95$^{cd}$ | 5.20$^{a}$ | 2.18$^{d}$ | 4.49$^{ab}$ | 2.68$^{cd}$ | 5.09$^{a}$ | 3.67$^{bc}$ | 4.56$^{ab}$ | 0.42 | < 0.01 |
| Ethoxyquin | | 0.04$^{b}$ | 0.00$^{b}$ | 0.32$^{a}$ | 0.36$^{a}$ | 0.08$^{b}$ | 0.00$^{b}$ | 0.29$^{a}$ | 0.31$^{a}$ | 0.07 | < 0.01 |

[a-d] Within a row, means without a common superscript differ at $P \leq 0.05$.

Running Head: Antioxidants and SR membrane instability

# Feeding vitamin E may reverse sarcoplasmic reticulum membrane instability caused by feeding wet distillers grains plus solubles to cattle

**M. D. Chao, K. I. Domenech-Perez, and C. R. Calkins[1]**

Department of Animal Science, University of Nebraska-Lincoln, Lincoln, NE 68583-0908

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[1]Corresponding author: Ccalkins1@unl.edu

ABSTRACT: Previous research showed feeding wet distillers grains plus solubles (WDGS) in beef feedlot diets increases polyunsaturated fatty acid (PUFA) concentration in the sarcoplasmic reticulum (SR) membrane, thereby altering membrane integrity, resulting in more rapid postmortem calcium leakage and improving tenderness through early activation of calcium dependent proteases. Supplementation with antioxidants may mitigate such effects. One hundred and sixty cross-bred cattle were finished on either corn or 30% WDGS with 4 antioxidant treatments [Vitamin E (E) at 22.5 mg (50 IU)/hd/d– control; E at 450 mg (1,000 IU)/hd/d; Agrado (AG) at 3 g/hd/d (215 mg/kg of feed); a combination of 225 mg (500 IU)/hd/d of E and 3 g/hd/d of AG]. Ten striploins from each treatment (n=80) were collected and aged for 2, 7, and 14 d and placed under retail display conditions for 0 and 7 d. Tenderness, free calcium concentrations, sarcomere lengths, proteolysis (troponin-T degradation), and SR membrane phospholipid, neutral lipid, total lipid and fatty acid profiles were measured. Feeding WDGS decreased ($P < 0.05$) proportions of 16:0, 16:1, 18:0, 18:1V and total saturated fatty acids and tended ($P < 0.1$) to decrease 18:1 and total monounsaturated fatty acids, but increased ($P < 0.05$) 15:0, 17:0, 18:1 trans, 18:2 and total PUFA in the SR membrane. Supplementing E decreased ($P < 0.05$) 18:2 and tended to decrease ($P = 0.09$) PUFA when compared to diets without antioxidants. Furthermore, steaks from steers fed WDGS without any supplementation had more ($P < 0.05$) phosphatidylcholine in the SR membrane, but less ($P < 0.05$) phosphatidylethanolamine compared to steaks from steers fed corn or WDGS supplemented with E. One interesting note is supplementing AG in the corn-only diet also created similar effects as the WDGS-only diet for SR membrane phospholipid profile. There were no differences in tenderness, sarcomere length or free calcium concentrations

among steaks from steer in any of the treatments, aging or display periods. At 2 d postmortem, steaks from steers fed WDGS had more troponin-T degradation compared to steaks from steers fed corn-only or either diet supplemented with E only. These results suggest that the alteration of SR membrane fatty acid profile in WDGS-fed cattle may affect the ratio of SR membrane phospholipids, thereby creating membrane instability and thus increasing proteolysis, while feeding E mitigates such effects.

Key words: Beef, distillers grains, antioxidants, SR membrane fatty acids, proteolysis, free calcium

## INTRODUCTION

Muscle is an elegant biological system with mechanisms in place to control calcium. After death, calcium ions diffuse out of the sarcoplasmic reticulum (SR) to the sarcoplasm either through the calcium release channels (Hidalgo et al., 2000) or channels formed by the degradation of SR membrane phospholipids (Ji and Takahashi, 2006). The presence of free sarcoplasmic calcium facilitates activation of the calpain enzyme system with subsequent proteolysis of muscle proteins and a consequent improvement in meat tenderness (Koohmaraie, 1994, 1992; Taylor and Koohmaraie, 1998).

Biological membranes are primarily comprised of a phospholipid bilayer (Singer and Nicolson, 1972), and phospholipids are known to contain a high proportion of PUFA to introduce kinks into the hydrocarbon chains in order to maintain membrane fluidity (Wood et al., 2008). Furthermore, diets rich in PUFA are known to increase the proportion of PUFA in phospholipids (Dannenberger et al., 2006; Enser et al., 2000) as

well as modify the phospholipid profile (Dannenberger et al., 2006; Mlekusch et al., 1993) in muscle tissue and thus result in more rapid lipid oxidation unless antioxidants are supplemented.

Senaratne (2012), Segers et al. (2011) and Depenbusch et al. (2009) all found that beef from cattle fed distillers grains was more tender than beef from steers fed corn, and both Senaratne (2012) and Secrist et al. (1995) found beef from cattle fed antioxidants was tougher than beef from steers that were not fed antioxidants. Perhaps, an increase of PUFA in SR membrane contributed to instability of the SR membrane, making it more susceptible to oxidation and thereby causing the early post-rigor release of calcium. Early calcium release would more quickly activate calcium-dependent calpains and thus improve beef tenderness. Conversely, antioxidants might reverse such effects by preserving stability of the SR membrane.

Feeding WDGS provides an excellent model to generate samples with varying degrees of SR membrane oxidation capacity, as does feeding different antioxidants. Vitamin E (E) is a membrane-associated antioxidant that can maintain cellular integrity and delay lipid and pigment oxidation effectively (Schaefer, 2007), and Agrado-Plus (AG) is a synthetic antioxidant supplement manufactured by Novus International, St. Louis, MO, which contains a mixture of ethoxyquin and TBHQ (tertiary-butylhydroquinone). A more thorough background of these antioxidants was discussed in (Manuscript 2). The objective of this study was to characterize the relationships among SR membrane oxidative capacity, postmortem calcium release and subsequent proteolysis.

## MATERIALS AND METHODS

All animal use protocols were approved by the University of Nebraska-Lincoln's Animal Care and Use Committee.

*Animals*

One hundred and sixty Continental X British cattle were blocked by BW, stratified by BW within each block, and assigned randomly to pens within block. Pens were randomly assigned to one of the eight treatments with two pens per treatment and ten cattle per pen. Cattle were fed for 106 d on either a corn-only diet or a corn-based diet with 30% WDGS (DM basis) with one of four antioxidant treatments. The eight treatments were 1) a corn-only diet with E supplementation at 22.5mg (50 IU)/hd/d (Control); 2) a corn-only diet with E supplementation at 450 mg (1,000 IU)/hd/d (Corn+E); 3) a corn-only diet with AG supplementation at 3 g/hd/d (215 mg/kg of feed) (Corn+AG); 4) a corn-only diet with a combination of 225 mg (500 IU)/hd/d of E and 3 g/hd/d (215 mg/kg of feed) of AG (Corn+E+AG); 5) a corn based diet with 30% WDGS with E supplementation at 22.5 mg (50 IU)/hd/d (WDGS); 6) a corn based diet with 30% WDGS with E supplementation at 450 mg (1,000 IU)/hd/d (WDGS+E); 7) a corn based diet with 30% WDGS with AG supplementation at 3 g/hd/d (215 mg/kg of feed) (WDGS+AG); 8) a corn based diet with 30% WDGS with a combination of 225 mg (500 IU)/hd/d of E and 3 g/hd/d (215 mg/kg of feed) of AG (WDGS+E+AG).

*Sample Collection, Fabrication and Preparation*

All cattle were harvested at a commercial abattoir. After 48 h of postmortem chilling, 80 carcasses were selected (10 carcasses from each treatment). Carcass selection

was based on treatment and quality grade (Choice). The strip loins were collected, vacuumed-packaged and transported to Loeffel meat laboratory at the University of Nebraska-Lincoln.

Strip loins from the left sides of the carcasses were fabricated at d 2 and 7 of postmortem aging, and strip loins from the right sides of carcasses were fabricated on d 14 of postmortem aging. Each strip loin was fabricated into 2 tenderness samples (2.54 cm) and 2 lab samples (1.27 cm) for each aging period from the anterior to the posterior end of the loin muscle. The remainders of the strip loins were immediately vacuum packaged (3mil STD barrier, Prime Source, St. Louis, MO) on a Multivac Packaging machine (MULTIVAC C500, Multivac Inc., Kansas City, MO) and aged to the next designated aging period.

Upon fabrication, steaks were overwrapped in Styrofoam trays (Styro-Tech, Denver, CO) with oxygen permeable polyvinyl chloride film (PSM18, Prime Source, St. Louis, MO) and subjected to retail display conditions (2 ± 2$^{o}$C, and exposed to continuous 1,000-1,800 lux warm white fluorescence lighting). For each aging period, samples for tenderness, free calcium concentrations and proteolysis were obtained on d 0 and 7 of retail display. Sarcomere length was determined on samples with 0 d retail display after 2 d of aging. For SR membrane fatty acid, phospholipid, neutral lipid and total lipid analyses, samples were obtained at d 0 of retail display after 14 d of aging. At the end of the allotted treatments, all samples were vacuum packaged and frozen at -20$^{o}$C (tenderness samples) or -80$^{o}$C (lab samples) until analyzed. The samples were pulverized in liquid nitrogen with a blender (model 51BL32, Waring Commercial, Torring, CT).

*Objective Tenderness (WBSF)*

Steaks were removed from the freezer and thawed at 4°C for 24 h prior to grilling. An insulated type T thermocouple (5SC-TT-T-30-120, OMEGA Engineering, Inc., Stamford, CT) was inserted into the geometric center of each steak and attached to an OMEGA 450-ATT thermometer (OMEGA Engineering, Inc., Stamford, CT) to monitor the internal temperature of the steak. Steaks were grilled on a Hamilton Beach Indoor-Outdoor grill (Model 31605A, Proctor-Silex, Inc., Washington, NC), flipped once when the internal temperature reached 35°C, and removed from the grill when they reached an internal temperature of 71°C. Grilled steaks were cooled at 4°C for 24 h, and six cores, 1.27 cm in diameter, were removed parallel to the muscle fibers using a drill press. Cores were sheared on a Texture Analyzer (model TMS-PRO, Food Technology Crop., Sterling, VA) with a Warner-Bratzler blade. The mean peak shear force (kg) of 6 cores was calculated for each steak.

*Free Calcium Concentration*

Free calcium was quantified using the procedure described by Parrish et al. (1981) with modifications. Three grams of powdered sample was centrifuged at 196,000 x *g* (Beckman L7-65 Ultracentrifuge with a SW55Ti rotor; Beckman Coulter, Brea, CA) at 4$^{o}$ C for 30 min. Seven hundred microliters of the supernatant was collected and treated with 0.1 mL of 27.5% trichloroacetic acid (TCA). Samples were centrifuged at 6,000 x *g* (Eppendorf model 5430; Eppendorf, Hamburg, Germany) for 10 min. Four hundred µL of supernatant was transferred to a syringe, and the volume was brought to 4 mL with deionized distilled water (dd$H_2O$). The diluted calcium sample was filtered through 13

mm diameter Millex-LG 0.20 μm syringe filters (Millipore, Bedford, MA). Calcium concentration of samples was quantified using an inductively-coupled plasma emission spectrometer (iCAP 6500 Radial; Thermo Electron, Cambridge, UK) with appropriate calcium concentration standards.

*Sarcomere Length*

Sarcomere length was determined using the powdered sample Helium-Neon laser method described by Cross et al. (1981) with modifications from Dolazza and Lorenzen (2014). Five individual sarcomeres per sample were determined, and the final sarcomere length was calculated according to the equation described by Cross et al. (1981).

*Proteolysis (Troponin-T degradation)*

Myofibrillar proteins were isolated according the procedure described by Pietrzak et al. (1997) with modifications. Three grams of powdered meat sample were suspended in ice-cold 10 mL rigor buffer (0.1 *M* KCl, 2 m*M* $MgCl_2$, 1 m*M* EGTA, and 10 m*M* $K_2HPO_4$) at pH 7.4 and homogenized using a Polytron homogenizer (model CH-6010; Kinematica, Luzern, Switzerland) at setting 6 for 15 s. The homogenate was filtered thorough doubled-layered cheese cloth to remove connective tissue and fat. One milliliter of homogenate was transferred and centrifuged at 4,000 x *g* for 5 min. The supernatant was decanted and the pellet was resuspended in 1 mL of ice-cold rigor buffer. The pellet washing step was repeated three times. One milliliter of extraction buffer (0.1 *M* Tris-HCl, 1.25 m*M* EDTA, 5% SDS) at pH 8 was added to the washed pellet and vortexed thoroughly. Protein concentration was determined using a Pierce bicinchoninic acid

(BCA) protein assay kit (Pierce Biotechnology, Rockford, IL). All myofibrillar protein samples were diluted to 2 mg/mL with $ddH_2O$.

Degree of proteolysis was measured by troponin-T (TNT) degradation. All of the following procedures were conducted at room temperature. Twenty-five microliters of the 2 mg/mL myofibrillar protein samples were mixed with 2x Laemmli sample buffer (65.8 m*M* Tris-HCl, 2.1% SDS, 26.3% glycerol, 0.01% bromophenol blue) with 5% β-mercaptoethanol at 1:1 ratio. All samples were heated at 95°C for 5 min. Five microliters Kaleidoscope Pre-stained Protein Standard and prepared myofibrillar protein samples (5 µg) were loaded on 4-20% Mini-PROTEAN TGX™ precast polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) using a Bio-Rad Mini-PROTEIN 2 Cells system (Bio-Rad Laboratories). The system was run at constant voltage of 200 V for 40 min with a running buffer consisted of 25 m*M* Tris-base, 192 m*M* glycine and 0.1% SDS (pH 8.3). Proteins in the gels were blotted to polyvinylidene difluoride (PVDF) membranes (0.45 µm, Immobilon-FL transfer membrane; Millipore) using a Bio-Rad Mini-Trans-Blot Electrophoretic transfer cell (Bio-Rad Laboratories) for 60 min at a constant voltage of 100 V with ice-cold transfer buffer (25 m*M* Tris-base, 192 m*M* glycine, 20% methanol, 0.1% SDS at pH 9.2). Membranes were blocked for 2 hr in Odyssey Blocking Buffer (LI-COR, Lincoln, NE) and incubated for 1 h in primary anti-TNT (JLT-12; Sigma-Aldrich, St. Louis, MO) antibody at a dilution of 1:10,000 in Odyssey blocking buffer containing 0.2% Tween-20. Membranes were washed 3 times with Tris Buffered Saline containing 0.2% Tween-20 for 15 min and incubated in IRDye 680LT Conjugated Goat Anti-Mouse IgG1 (LI-COR) secondary antibody at a dilution of 1:10,000 in Odyssey blocking buffer containing 0.2% Tween-20 for 1 h. Membranes were washed 3 times and scanned using

Odyssey Infrared Imaging system (LI-COR) at 700 nm.

All intact TNT and degraded TNT products were measured by quantifying band intensities (k. pixels) using Odyssey application software version 1.1. Bands 1 and 2 (38 and 35 kD, respectively) corresponded to intact TNT while bands 3 and 4 (30 and 28 kD, respectively) correspond to degraded TNT. Percent TNT degraded was measured by band intensities of degraded bands divided by band intensities of all bands in a specific lane.

*SR Membrane Extraction*

The SR membrane was isolated according to the procedure described by Hemmings (2001) with modifications. Ten grams of powdered samples were suspended in ice-cold 35 mL homogenization buffer (10 m*M* $NaHCO_3$, 2 m*M* sodium azide, 10 m*M* Tris-Cl, and 1 m*M* dithiothreitol) at pH 7.5 and homogenized using a Polytron homogenizer (Kinematica) at setting 6 for 15 s. Homogenate was transferred into a 50 mL plastic centrifuge tube and centrifuged at 2000 × *g* for 10 min at 4$^{o}$C. The supernatant was collected and centrifuged at 10,000 x *g* (Sorvall RC5B Superspeed Centrifuge; Thermo Scientific, Rockford, IL) for 30 min at 4$^{o}$C. Four and a half percent of KCl (w/v) was added to the supernatant and stirred for 30 min on ice. The supernatant was centrifuged at 100,000 x *g* for 60 min at 4$^{o}$C (Beckman L7-65 Ultracentrifuge with a SW28 rotor; Beckman Coulter). The final supernatant was discarded, and the pellet was resuspended in 1 mL of 10mM tris buffer and stored in -80$^{o}$C until use.

*SR Membrane Fatty Acids*

For SR membrane fatty acids, total lipid was extracted following the procedure of

Bligh and Dyer (1959). After extraction, lipids were converted to fatty acid methyl esters according to Morrison and Smith (1964) and Metcalfe et al. (1966). The prepared fatty acid methyl esters were further concentrated by drying at 60°C under constant nitrogen gas purging and mixed with 100 μL of hexane. The fatty acid methyl esters were transferred to 100 μL spring bottom vial inserts and inserted into the GC vials. The samples were analyzed using gas chromatography (Hewlett-Packard 6890 FID GC System; Agilent Technologies, Santa Clara, CA) with setups described by Mello et al. (2012).

*SR Membrane Phospholipid, Neutral Lipid and Total Lipid*

Total lipids were extracted from a second set of SR membrane samples. Thirty microliters of 2% methanol and 1 % dd$H_2O$ in chloroform was added to each lipid sample. For SR membrane phospholipid, neutral lipid and total lipid profile, samples were separated into 10 different lipid groups by one-dimensional thin-layer chromatography (TLC) described by Leray et al. (1987) with modifications. Briefly, Whatman LK5 TLC plates (Whatman, Clifton, New Jersey) were prewashed by migration up to 1 cm from the top with chloroform/methanol (1/1, v/v) and wetted thoroughly with 2.3% (w/v) boric acid solution. All 30 μL of each extracted lipid sample was deposited on the plate and placed in the chromatography tank containing the following solvent: chloroform/ethanol/dd$H_2O$/triethylamine (30/35/7/35, v/v). After the migration was complete, lipid and phospholipid spots were quantified by the method described by Baron and Coburn (1984) with modifications. Each plate was dampened with a 10 % (w/v) cupric sulfate solution in 8% (w/v) phosphoric acid and placed in an

oven at 180°C for 10-15 min. The isolated fractions were identified by comparing their $R_f$ values with known lipid standards. The plate was scanned by a desktop scanner (Artisan 730, Epson, Nagano, Japan) and the isolated fractions were quantified using Quantity One 1-D Analysis Software (Bio-Rad). Each phospholipid or neutral lipid was measured as a percentage of total phospholipids or neutral lipid in one lane. For total lipid profiling, all phospholipids and all neutral lipids from one lane were combined as one class. Each lipid class was measured as a percentage of total lipids on one specific lane.

*Statistical Analysis*

Data for free calcium concentration and TNT degradation were analyzed as a split-split plot design with dietary treatments as the whole-plot, aging period as the split-plot and retail display time as the split-split plot. Sarcomere length, SR membrane fatty acid, phospholipid, neutral lipid and total lipid profiles were analyzed as a completely randomized design. Data were analyzed using the GLIMMIX procedure of SAS (version 9.2, Cary, NC, 2009). Separation of means was conducted using LSMEANS procedure with PDIFF or SLICEDIFF options at $P < 0.05$. In addition, the CONTRAST statements in SAS were used to compare effects of feeding Corn vs. WDGS, E vs. no E, AG vs. no AG and E+AG vs. no E+AG.

## RESULTS

The SR membrane fatty acid profiles are shown in Table 1. Fatty acid analysis revealed distinct differences among fatty acids profile of SR membrane from cattle fed different diets. The SR membrane from steers fed WDGS had higher ($P < 0.05$)

concentrations of 15:0, 17:0, 18:1 trans, 18:2 and total PUFA, lower concentrations of ($P < 0.05$) 16:0, 16:1, 18:0, 18:1V and total saturated fatty acids and tended ($P < 0.10$) to have lower concentrations of 18:1 and total monounsaturated fatty acids compared to SR membrane from cattle fed corn-only. Feeding E decreased ($P < 0.05$) SR membrane 18:2 and tended ($P < 0.10$) to decrease SR membrane PUFA when compared to diets without any antioxidants. Long and very long chain PUFAs like 20:3, 20:4, 20:5, 22:4, 22:5 in the SR membrane were not affected by the treatments.

The SR membrane phospholipid, neutral lipid and total lipid profiles are shown in Table 2. The WDGS treatment increased ($P < 0.05$) concentration of phosphatidylcholine (PC) and decreased ($P < 0.05$) concentration of phosphatidylethanolamine (PE) in the SR membrane phospholipids compared to the control, Corn+E and WDGS+E treatments. It is interesting to note that the Corn+AG treatment created similar effects as the WDGS treatment for the SR membrane phospholipid profile, while such effects were not observed for the WDGS+AG treatment. No apparent shift in phospholipid profile was observed when E was combined with AG. Furthermore, the Corn+AG treatment tended ($P < 0.10$) to have more lysophosphatidylcholine when compared to Corn+E treatment. No differences ($P > 0.10$) in total phospholipids, mono-, di- and triacylglyceride, cholesterol, free fatty acids and total neutral lipid among treatments were observed.

Results of TNT degradation are shown in Table 3. An example of a Western blot used to quantify TNT degradation is shown in Figure 1. There was a significant ($P < 0.01$) interaction between aging and treatments for TNT degradation. The WDGS treatment increased ($P < 0.05$) TNT degradation compared to the control and Corn+E, and tended to increase ($P < 0.10$) TNT degradation compared to WDGS+E treatment

after 2 d of aging. In addition, the AG treatment increased ($P < 0.05$) TNT degradation for the corn-only diet, but not for the WDGS diet, after 2 d of aging. When E was combined with AG, no effect of E or AG on TNT degradation was observed. It is worth noting that the LS means for TNT degradation at 2 d aging is highly correlated with the LS means for SR membrane phospholipid PC ($R^2 = 0.90$) and PE ($R^2 = 0.88$) of the treatments (Figure 2).

Sarcomere length data are shown in Table 3. There were no differences ($P > 0.10$) in sarcomere length among steaks from cattle in any of the treatment, and there were also no differences ($P > 0.10$) in WBSF among steaks from cattle on any of the treatments in any of the aging and display periods (data not shown). Finally, there was only a significant ($P < 0.05$) age by display interaction for free calcium concentration (Table 4). Treatment did not have any effect on the free calcium concentration. However, there was a clear pattern that free calcium concentration decreased ($P < 0.05$) over aging and retail display.

## DISCUSSION

When compared the beef SR membrane fatty acid profiles from this study to the beef muscle tissue fatty acid profiles from the same set of cattle (Table 1 in manuscript 2), there were similarities in trend, but the magnitude of individual fatty acid concentration is vastly different. In general, the PUFA for SR membrane fatty acids is 4X more than the PUFA content in muscle tissue. The saturated fatty acids (SFA) for SR membrane are 30% less than the SFA in muscle tissue, particularly for short chain SFA, and the MUFA for SR membrane fatty acids is also 10% less than the MUFA for muscle

tissue. This phenomenon was explained thoroughly in manuscript 1 that high concentrations of PUFA are associated with phospholipids to maintain membrane fluidity (Wood et al., 2008), and SFA is less desired because the tight packing of these straight chain lipids tend to decreased fluidity (Cooper, 2000).

The SR membrane fatty acids results from this study also echo the results of Chao et al. (2015) for beef SR membrane fatty acid profiles in that all individual fatty acid concentrations are within reasonable range from one study to another. However, endoplasmic reticulum (ER) and SR membrane fatty acid profiles from rat liver (Tahin et al., 1981) and mouse muscle (Birkle et al., 1993) are slightly different from the SR membrane fatty acids profile from beef muscle of this study. The rat and mouse ER and SR membrane from those studies had significantly more 20:4 or 22:6 than the membrane of this study. The differences in SR membrane fatty acids profile can easily be attributed to diet, species and location differences. As discussed in manuscript 1, the modification of beef SR membrane fatty acid profiles by feeding WDGS occurs by the same mechanism that modification of beef muscle fatty acid profiles occurs. For monogastrics, dietary PUFAs do not have to go through biohydrogenation in the rumen and thus can be more easily incorporated into cell or organelle membranes (Wood et al., 2008).

One interesting but unexpected observation from this study was that E supplementation decreased SR membrane 18:2 concentrations compared to diets without any antioxidant supplementation. This phenomenon was not observed in the muscle tissue fatty acid profile from the same set of cattle (manuscript 2), nor was it observed in many studies on alteration of muscle tissue fatty acid profile by E supplementation (Álvarez et al., 2008; de Mello, 2010; Kasapidou et al., 2009). Unfortunately, because of

the uniqueness of this particular study, no supporting evidence to the author's knowledge can be found on the manipulation of SR membrane fatty acid profile by antioxidants. Pirozhkov et al. (1992) showed that E supplementation can prevent the ethanol-induced increase of extra-long chain PUFA in mice myocardium by reversing the inhibitory influence of alcohol on delta-5-desaturase. Furthermore, Century and Horwitt (1964) found that 18:3 in cod liver oil diet inhibited the conversion of 18:2 to 20:4 in E-deficient chicks, but E supplementation prevented such inhibition. Perhaps, E is necessary for the biosynthesis of 20:4 from 18:2 or it promotes the activity of delta-5-desaturase. Either way, it would result in an increased utilization of 18:2, which would have decreased 18:2 concentrations in this study. Conversely, high doses of E are known to alleviate oxidative stress of rumen microbes (Tagliapietra et al., 2013). Therefore, supplementation of E in this study might result in a more efficient fermentation process, thus retaining less 18:2 from the diet for membrane incorporation.

Results from this study showed that a diet high in PUFA increased SR membrane proportion of PC, but decreased PE, while supplementation of E mitigated such effect. Mlekusch et al. (1993) found the same shift in rat liver when rats were fed a diet high in PUFA (corn oil). The β-position of both PC and PE are highly preferential for PUFA (Söderberg et al., 1991); however, more dietary fatty acids are known to incorporate into PC than PE because PC is the only phospholipid that undergoes transacylation by plasma fatty acids (Van Deenen et al., 1963). Beare and Kates (1964) further supported the statement by showing rat muscle PC can incorporate dietary 18:2 better than PE can. This inhibition of a shift from PE to PC as a result of E supplementation can probably be linked back to the decrease of 18:2 in SR membrane fatty acid profile from E

supplementation. However, a shift of PE to PC without the 18:2 alterations was also observed for the Corn+AG treatment. Therefore, there are likely other factors contributing to this phenomenon.

Another hypothesis regarding the mechanism of the shift is that PUFA can upregulate two key genes in PC synthesis and PE to PC conversion: choline-phosphate cytidylyltransferase A (Pcyt1a) and phosphatidylethanolamine N-methyltransferase (Pemt) (Park et al., 2014). Fu et al. (2011) further demonstrated that sarco-endoplasmic reticulum calcium ATPase (SERCA) oxidative stress indicators such as the expression of ER stress-inducible protein was reduced upon the suppression of Pemt, linking such conversion of phospholipid profile to ER stress. Feeding a diet high in PUFA like WDGS might induce SR oxidative stress in this study, while supplementing E likely alleviated such stress on the SR thus inhibited the up-regulation of Pcyt1a and Pemt from the WDGS diets. Finally, Jacob and Lux (1968) showed that PE content was dramatically decreased in E-deficient rat red blood cells when exposed to reactive oxygen species (ROS) such as hydrogen peroxide. However, the proportion of PE was unchanged in E-supplemented rats. Phospholipid PE tends to contain higher proportions of endogenous extra-long chain PUFA than PC (Vance and Tasseva, 2013). It is possible that the higher proportions of extra-long chain PUFAs in PE were more prone to oxidation, and thus accelerated PE degradation, which contributed to the shift in SR membrane phospholipid profile. On the other hand, E supplementation suppressed the initiation of lipid oxidation, which in turn, could have preserved the full functionality of SERCA. Furthermore, formation of free radicals can occur when ethoxyquin and TBHQ are administered in high concentrations (>150 mg/kg of feed). Other factors like presence of transition metals

and change in solubility and pH can also convert these antioxidants to pro-oxidants (Sakihama et al., 2002; Skaare and Henriksen, 1975). These phenolic antioxidants can be converted to phenxyl radicals under different conditions and contribute short or long term toxicities to animals or people consuming meat from these animals (Sakihama et al., 2002). Perhaps, AG not only failed to act as an antioxidant, but transitioned into pro-oxidants, which stimulated oxidative stress and led to rapid decomposition of PE in SR membrane for cattle fed a corn-only diet.

There is a very good linear relationship between the LS means of TNT degradation at 2 d aging and the LS means of SR membrane PC and PE. The coefficients of determination ($R^2$ x 100) for TNT degradation and SR membrane PC and PE concentrations are 90 and 88, respectively, which indicates that both SR membrane PC and PE are likely good predictors for early proteolysis. Phospholipid PE plays an important role in the function of SERCA because PE binds to the transmembrane helices of the SERCA, and it is replaced by calcium when calcium is bound to the protein (Hunter et al., 1999). It has been shown many times that an increased PC to PE ratio in the SR membrane inhibits the calcium transport activity of SERCA (Cheng et al., 1986; Fu et al., 2011; Hunter et al., 1999; Li et al., 2004), which likely leads to free calcium accumulation in sarcoplasm early postmortem because of less functional SERCA along with the depletion of ATP sources. The ability of E to prevent such a shift maintains SERCA function for cattle from WDGS+E treatment, while AG can induce such a shift even with diets low in PUFA.

Moreover, Dannenberger et al. (2006) showed that a pasture diet high in PUFA significantly increased lysophosphatidylcholine (LPC) in the muscle tissue of German

Simmental bulls compared to the muscle tissue from animals fed a concentrate diet. Although the diet didn't have any effect on LPC in this study, the Corn+AG treatment tended ($P$ = 0.09) to increase this product of phospholipid hydrolysis compared to Corn+E treatment. Lysophospholipids are capable of increasing membrane permeability through stabilizing dimer formation between two peptide helices to form open channels (Lundbaek and Andersen, 1994). Flemming et al. (2006) showed that lysophospholipids can activate transient receptor potential channels (permeable to calcium, sodium and potassium ions) by supporting the channel's structure. This finding again provided evidence that AG acted as a pro-oxidant in the corn diet, thus increased the oxidation rate of membrane phospholipids, leading to an increased proportion of LPC. However, the pro-oxidation factors for AG are not present in the WDGS diet as the LPC level in WDGS+AG was very low.

The TNT degradation results indicate that the calpain activity was distinctly different among treatments at 2 d aging. The WDGS treatment had more TNT degraded compared to the control, Corn+E and WDGS+E treatments. Unexpectedly, the AG treatment seemed to act as a proteolytic catalyst. It not only failed to inhibit proteolysis, but improved proteolysis for the corn-only diet. However, the effect was not observed for the WDGS+AG treatment. These early proteolytic differences are likely attributed to possible differences in pre-rigor free calcium concentrations due to loss of SERCA functionality from the PE to PC conversion as discussed earlier. Jaime et al. (1992) showed that a more intense proteolysis occurred in cold-shortened lamb muscle compared to normal lamb muscle due to higher concentrations of free calcium pre-rigor and early post-rigor. However, sarcomere length is established as a marker for pre-rigor

free calcium level (Pearson et al., 1973), and this study showed no differences in sarcomere length among the treatments. The extent of rigor shortening has been reported to vary due to temperature, pH and ATP reserves (Busch et al., 1967; Honikel et al., 1983). Koohmaraie et al. (1998) showed that freezing carcasses immediately after slaughter and then storing at just below freezing for a period of time to depleted ATP and can prevent thaw rigor. Since samples from this study were collected at a major packing plant, low voltage electrical stimulation was applied to each carcass to deplete ATP to prevent cold shortening. This practice might have resulted in the unchanged sarcomere length with evidence indicating different concentration of pre-rigor and early post-rigor free calcium.

This is not the first time that a poor correlation was found between free calcium concentration and tenderness/or proteolysis (Miller et al., 2011; Parrish et al., 1981). However, this is the first time to the author's knowledge that free calcium concentration was found to decrease over time post rigor. Many studies reveal an upward trend over time for postmortem free calcium concentration. However, the results varied. Ji and Takahashi (2006) reported that in rabbit, free calcium concentration increased from 33 μM to 230 μM at 30 hours postmortem; and in beef and pork, free calcium increased from 16 μM to 210 μM at 3 d postmortem. Hopkins and Thompson (2001) documented that free calcium plateaued at 110 μM after 2 d postmortem in lamb. Finally, Senaratne (2012) showed that free calcium increased from 791 to 947 μM from 8 d aging to 28 d aging in beef. Dransfield (1999) noted many of the inconsistencies in the literature and questioned whether the concentrations of free calcium really change post rigor.

Most of these divergent results can be explained by the different methodologies

used to measure the concentration of “free” sarcoplasmic calcium in muscle. This study and Senaratne (2012) both treated the samples with 27.5 % TCA and quantified calcium concentrations using an inductively-coupled plasma emission spectrometer, while Ji and Takahashi (2006) and Parrish et al. (1981) used an atomic absorption spectrophotometer instead. Hopkins and Thompson (2001) used calcium sensitive electrodes on samples that were not treated with TCA; and finally, Miller et al. (2011) did not specify the methodology used in their study. The downward trend of free calcium concentration found in this study might be attributed to purge loss during aging and display. One of the origins for purge loss is the intracellular space of muscle cells - the sarcoplasm (Huff-Lonergan and Lonergan, 2005). As structural proteins degrade and membrane permeability increases due to oxidation during aging and display, it makes sense that a portion of the free calcium is being flushed out with the purge. This study implied that a difference existed in pre-rigor free calcium concentration; unfortunately, collecting samples pre-rigor was not an option for this study.

Finally, a difference in objective tenderness and was expected as previous work from our lab has shown steaks from cattle fed WDGS were more tender than steaks from cattle fed corn or WDGS with dietary antioxidants (Chao et al., 2015; Senaratne, 2012), and differences in TNT degradation from this study were also observed. However, these WDGS or antioxidant effects on tenderness were not observed in every study. Perhaps, such subtle differences in tenderness can only be detected using extremely sensitive methodologies (SDS gel electrophoresis and Western blotting).

# CONCLUSION

The phospholipid profile shift and TNT degradation from this study provided strong evidence that levels of free calcium pre-rigor or early post-rigor were different among the treatments. Three mechanisms that might contribute to these differences were speculated. First, the shift of SR membrane fatty acid composition from different diets and antioxidants caused a shift of SR membrane phospholipid profile as PC is the preferred form of dietary PUFA. Second, the high PUFA diet upregulated the PC synthesis genes, and E down-regulated the genes through alleviating oxidative stress, while AG up-regulated the genes through possible conversion to pro-oxidants. Third, ROS selectively attacked PE rather than other major phospholipids, leading to the shift of SR membrane phospholipids. Again, E can quickly terminate the oxidation, while AG has minimum antioxidant effects, and may even act as a pro-oxidant when added to the corn-only diet. This possible genetic/chemical/dietary-induced phospholipid shift might shed light on the mechanism of meat tenderization, and future research is needed to fully understand the role of antioxidants on this diet induced phospholipid shift.

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Figure 1. Representative examples of Western blots that were used to quantify troponin-T (TNT) degradation. Lane a, muscle aged 2 d with 0 d retail display. Lane b, muscle aged 2 d with 7 d retail display. Lane c, muscle aged 7 d with 0 d retail display. Lane d, muscle aged 7 d with 7 d retail display. Lane e, muscle aged 14 d with 0 d retail display. Lane f, muscle aged 14 d with 7 d retail display.

Figure 2. Relationship of the LS means of troponin-T (TNT) degradation (muscle aged 2 d) with a) LS means of phosphatidylethanolamine (PE) and b) LS means of phosphatidylcholine (PC) in total sarcoplasmic reticulum (SR) membrane phospholipid.

Figure 1.

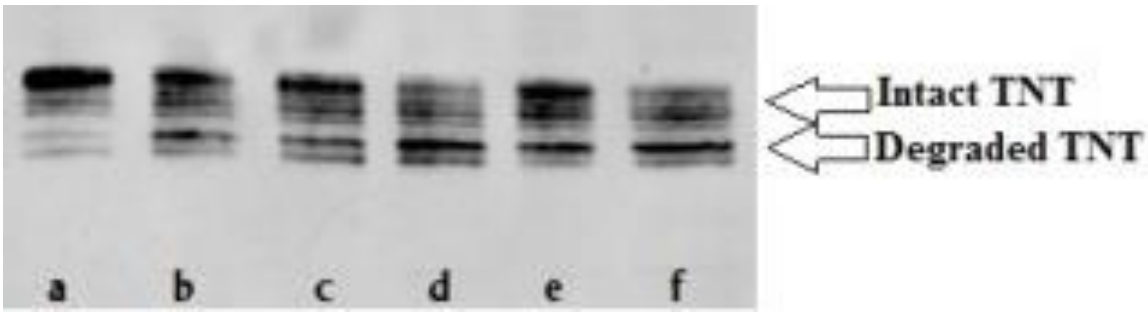
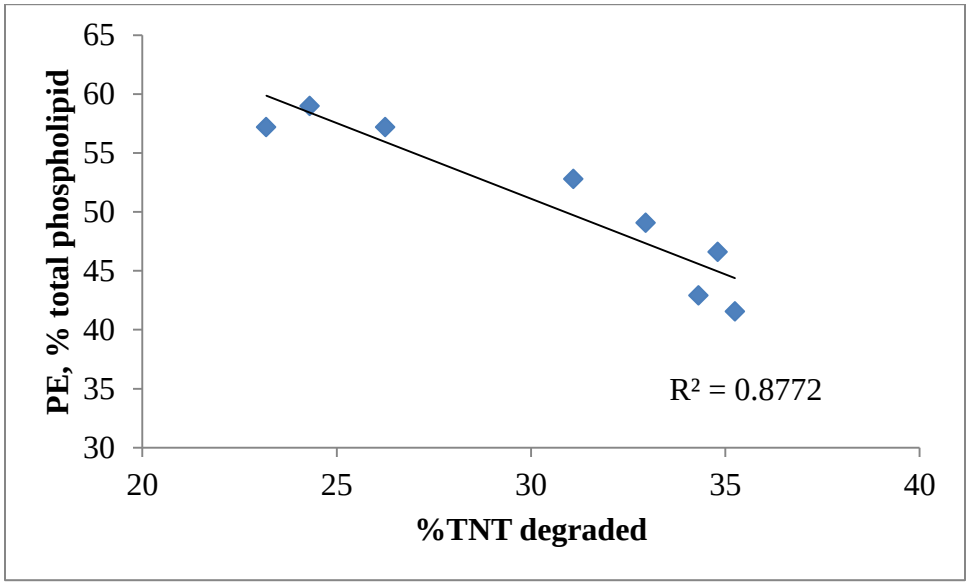


Figure 2.

(a)


PE, % total phospholipid
65
60
55
50
45
40
35
30
20
25
30
35
40
%TNT degraded
R² = 0.8772

(b)

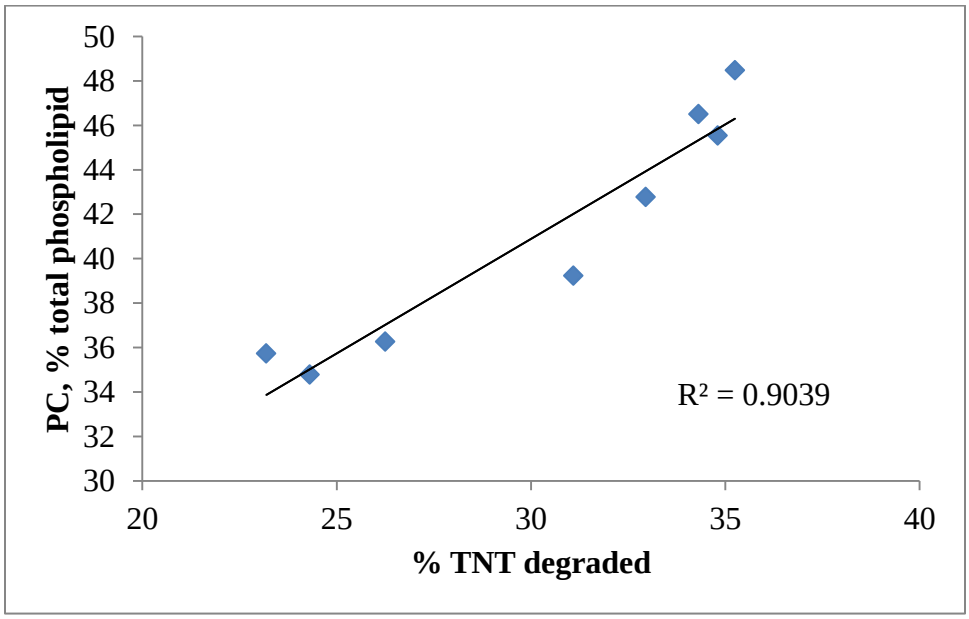

PC, % total phospholipid
50
48
46
44
42
40
38
36
34
32
30
20
25
30
35
40
% TNT degraded
R² = 0.9039

Table 1. Fatty acid profiles (%) of sarcoplasmic reticulum membranes from strip loins (*m. longissimus lumborum*) from steers fed corn only or corn with wet distillers grains plus solubles (WDGS) supplemented with or without vitamin E (E) or Agrado (AG) or a combination of E and AG.

| | Dietary treatments | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Fatty Acids | Corn | Corn+E | Corn+AG | Corn+E+AG | 30%WDGS | WDGS+E | WDGS+AG | WDGS+E+AG | SEM | *P*-value |
| 14:0 | 0.66$^{ab}$ | 0.69$^{ab}$ | 1.25$^{a}$ | 1.30$^{a}$ | 0.89$^{ab}$ | 1.05$^{ab}$ | 0.59$^{ab}$ | 0.45$^{b}$ | 0.18 | < 0.01 |
| 15:0 | 0.30$^{ab}$ | 0.25$^{b}$ | 0.39$^{ab}$ | 0.42$^{ab}$ | 0.47$^{a}$ | 0.43$^{ab}$ | 0.41$^{ab}$ | 0.33$^{ab}$ | 0.04 | < 0.01 |
| 16:0 | 20.52$^{abc}$ | 18.71$^{bc}$ | 21.98$^{ab}$ | 22.14$^{a}$ | 20.65$^{abc}$ | 19.83$^{abc}$ | 19.26$^{abc}$ | 17.84$^{c}$ | 0.79 | < 0.01 |
| 16:1 | 2.23$^{abc}$ | 2.10$^{bc}$ | 3.03$^{a}$ | 2.81$^{ab}$ | 1.76$^{c}$ | 2.01$^{bc}$ | 1.81$^{c}$ | 1.70$^{c}$ | 0.21 | < 0.01 |
| 17:0 | 0.89$^{b}$ | 0.83$^{b}$ | 1.03$^{ab}$ | 0.98$^{ab}$ | 1.36$^{a}$ | 1.24$^{ab}$ | 1.15$^{ab}$ | 1.09$^{ab}$ | 0.10 | < 0.01 |
| 17:1 | 0.92 | 0.84 | 1.09 | 1.08 | 1.20 | 1.21 | 1.12 | 1.06 | 0.10 | 0.14 |
| 18:0 | 10.22 | 10.79 | 10.62 | 9.41 | 9.86 | 10.13 | 9.43 | 9.30 | 0.42 | 0.09 |
| 18:1T | 1.90$^{bcd}$ | 1.43$^{d}$ | 2.03$^{abcd}$ | 1.71$^{cd}$ | 2.77$^{ab}$ | 2.79$^{a}$ | 2.29$^{abc}$ | 2.42$^{abc}$ | 0.20 | < 0.01 |
| 18:1 | 30.45 | 32.08 | 35.60 | 31.55 | 26.45 | 31.15 | 27.68 | 27.31 | 2.12 | 0.06 |
| 18:1V | 2.17$^{ab}$ | 2.14$^{ab}$ | 2.38$^{a}$ | 2.27$^{ab}$ | 2.05$^{ab}$ | 2.07$^{ab}$ | 1.99$^{b}$ | 2.03$^{b}$ | 0.08 | 0.01 |
| 18:2 | 13.82$^{ab}$ | 11.58$^{b}$ | 11.12$^{b}$ | 13.81$^{ab}$ | 19.54$^{a}$ | 15.66$^{ab}$ | 17.55$^{a}$ | 18.61$^{a}$ | 1.30 | < 0.01 |
| 20:1 | 0.52 | 0.49 | 0.46 | 0.55 | 0.41 | 0.41 | 0.47 | 0.42 | 0.04 | 0.23 |
| 20:3 | 1.16 | 1.17 | 1.01 | 1.26 | 1.04 | 0.94 | 1.21 | 1.09 | 0.14 | 0.74 |
| 20:4 | 4.55 | 3.99 | 3.30 | 4.61 | 3.78 | 3.51 | 4.51 | 4.05 | 0.54 | 0.56 |
| 20:5 | 0.43 | 0.47 | 0.38 | 0.44 | 0.35 | 0.35 | 0.46 | 0.39 | 0.06 | 0.75 |
| 22:4 | 0.56 | 0.51 | 0.41 | 0.49 | 0.44 | 0.37 | 0.58 | 0.49 | 0.07 | 0.34 |
| 22:5 | 1.08 | 0.93 | 0.72 | 0.98 | 0.80 | 0.69 | 1.03 | 0.85 | 0.12 | 0.23 |
| SFA | 32.60$^{abc}$ | 31.15$^{abc}$ | 35.16$^{a}$ | 34.26$^{ab}$ | 33.24$^{ab}$ | 32.68$^{abc}$ | 30.78$^{bc}$ | 28.90$^{c}$ | 0.93 | < 0.01 |
| MUFA | 38.31 | 39.16 | 44.69 | 40.28 | 34.85 | 39.84 | 35.39 | 34.99 | 2.48 | 0.09 |
| PUFA | 21.60$^{ab}$ | 18.56$^{ab}$ | 16.78$^{b}$ | 21.59$^{ab}$ | 25.91$^{a}$ | 21.45$^{ab}$ | 25.14$^{a}$ | 25.39$^{a}$ | 2.12 | 0.03 |

[1]SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, and PUFA = polyunsaturated fatty acids.

[a-d] Within a row, means without a common superscript differ at $P \leq 0.05$.

Table 2. Phospholipid, neutral lipid and total lipid profiles of sarcoplasmic reticulum membrane from strip loins (*Longissimus lumborum*) from steers fed corn only or corn with wet distillers grains plus solubles (WDGS) supplemented with or without vitamin E (E) or Agrado (AG) or a combination of E and AG.

| | Dietary treatments | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Corn | Corn+ E | Corn+ AG | Corn+E+ AG | 30% WDGS | WDGS+ E | WDGS+ AG | WDGS+E+ AG | SEM | *P*-value |
| Phospholipids, % | | | | | | | | | | |
| Phosphatidylcholine | 35.74$^{cd}$ | 34.79$^{d}$ | 46.51$^{ab}$ | 39.24$^{abcd}$ | 48.48$^{a}$ | 36.27$^{bcd}$ | 42.78$^{abcd}$ | 45.54$^{abc}$ | 3.70 | 0.05 |
| Phosphatidyl-ethanolamine | 57.19$^{ab}$ | 58.98$^{a}$ | 42.90$^{c}$ | 52.78$^{abc}$ | 41.54$^{c}$ | 57.20$^{ab}$ | 49.06$^{abc}$ | 46.61$^{bc}$ | 4.13 | 0.02 |
| Phosphatidylinositol | 1.04 | 0.66 | 3.26 | 1.57 | 1.76 | 1.12 | 1.14 | 1.46 | 0.75 | 0.37 |
| Phosphatidylserine | 0.53 | 0.38 | 0.77 | 0.28 | 0.31 | 0.31 | 0.32 | 0.33 | 0.18 | 0.22 |
| Sphingomyelin | 5.16 | 5.40 | 5.36 | 5.28 | 7.39 | 4.62 | 6.48 | 5.67 | 1.11 | 0.74 |
| Lysophosphatidyl-choline | 0.34 | 0.12 | 1.19 | 0.85 | 0.49 | 0.48 | 0.22 | 0.39 | 0.24 | 0.09 |
| Neutral lipids, % | | | | | | | | | | |
| Mono, Di & Triacylglyceride | 95.61 | 97.74 | 92.69 | 96.08 | 95.03 | 94.47 | 96.77 | 95.00 | 1.79 | 0.42 |
| Cholesterol | 3.69 | 1.97 | 4.36 | 2.36 | 4.11 | 3.51 | 2.52 | 3.39 | 1.04 | 0.61 |
| Free Fatty Acids | 0.71 | 0.29 | 2.93 | 1.56 | 1.42 | 2.01 | 0.71 | 1.61 | 0.84 | 0.51 |
| Lipid, % | | | | | | | | | | |
| Phospholipids | 50.28 | 50.89 | 58.86 | 46.93 | 52.64 | 59.36 | 51.44 | 51.90 | 4.16 | 0.43 |
| Neutral Lipid | 49.72 | 49.11 | 41.14 | 53.07 | 47.36 | 40.64 | 48.56 | 48.10 | 4.16 | 0.43 |

[a-d] Within a row, means without a common superscript differ at $P \leq 0.05$.

Table 3. Troponin-T (TNT) degradation and sarcomere length of strip loins (*Longissimus lumborum*) from steers fed corn only or corn with wet distillers grains plus solubles (WDGS) supplemented with or without vitamin E (E) or Agrado (AG) or a combination of E and AG.

| | | Dietary treatments | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aging (d) | Corn | Corn+E | Corn+ AG | Corn+E+ AG | 30% WDGS | WDGS+ E | WDGS+ AG | WDGS+E +AG | SEM | *P*-value |
| TNT, % Degraded | | | | | | | | | | 4.08 | 0.05 |
| | 2 | $23.19^{Ab}$ | $24.31^{Ab}$ | $34.31^{Aa}$ | $31.09^{Aab}$ | $35.25^{Aa}$ | $26.25^{Aab}$ | $32.95^{Aab}$ | $34.81^{Aa}$ | | |
| | 7 | $39.91^{B}$ | $41.30^{B}$ | $47.98^{B}$ | $43.00^{B}$ | $45.70^{B}$ | $40.31^{B}$ | $43.64^{B}$ | $45.02^{B}$ | | |
| | 14 | $44.71^{C}$ | $48.91^{C}$ | $55.03^{C}$ | $46.08^{B}$ | $53.80^{C}$ | $48.77^{C}$ | $53.18^{C}$ | $52.33^{C}$ | | |
| Sarcomere Length, μm | | 1.79 | 1.72 | 1.74 | 1.80 | 1.78 | 1.75 | 1.77 | 1.74 | 0.02 | 0.24 |

$^{a-b}$ Within a row, means without a common superscript differ at $P \leq 0.05$.

$^{A-C}$ Within a column, means without a common superscript differ at $P \leq 0.05$.

Table 4. Free calcium concentrations (μ*M*) of strip loins (*Longissimus lumborum*) over aging and retail display d.

| | Retail display, d | | | |
|---|---|---|---|---|
| | 0 | 7 | SEM | *P*-value |
| Aging, d | | | 18.07 | 0.05 |
| 2 | 1156.88$^{Aa}$ | 1026.71$^{Ab}$ | | |
| 7 | 1149.91$^{Aa}$ | 978.75$^{Ab}$ | | |
| 14 | 984.02$^{Ba}$ | 898.77$^{Bb}$ | | |

$^{a-b}$ Within a row, means without a common superscript differ at $P \leq 0.05$.

$^{A-B}$ Within a column, means without a common superscript differ at $P \leq 0.05$.

# RECOMMENDATIONS FOR FUTURE RESEARCH

There are still many questions that needed to be answered for this research. First, a clear trend for changes in postmortem sarcoplasmic free calcium concentration has not been well established. As mentioned in my third manuscript, most of these divergent results can be explained by the different methodologies used to measure the concentration of "free" calcium in muscle. The studies from this dissertation and Senaratne (2012) both treated the samples with 27.5% trichloroacetic acid (TCA) and quantified sample calcium concentrations using an inductively-coupled plasma emission spectrometer (ICP), while Ji and Takahashi (2006) and Parrish et al. (1981) used an atomic absorption spectrophotometer (AA) on TCA-treated samples. Although both ICP and AA are well documented methods to measure calcium ions in a solution, TCA is known to dissociate bound calcium from proteins during the denaturation process (Rodríguez Rodríguez et al., 2001). In fact, TCA is widely used in studies to cleave bound calcium from protein bonds (Silva et al., 2001). After a close examination of my methodology, I have a strong feeling that my free calcium determination methodology is not truly determining "free" calcium, but instead, "total" calcium due to the release of bound calcium from calcium binding proteins like calsequestrin, sarcalumenin, histidine-rich calcium-binding protein, junctate and many others. However, both ICP and AA require atomization of the solution for absorption of optical radiation by free ions in the gaseous state, so the proteins have to be removed before injection, or the proteins can create interference and clog up the equipment (Endres and Rude, 1999; Hou and Jones, 2000). Therefore, ICP and AA should be excluded from future studies of "free" calcium. The correct method to measure free calcium concentration should be non-invasive (to maintain protein-calcium bonds)

with high selectivity and sensitivity for calcium ions. Jeacocke (1993) injected the calcium ion binding dye arsenazo III into muscle to determine mouse muscle free calcium concentration at rigor using a spectrophotometer, while Hopkins and Thompson (2001) used calcium-sensitive electrodes on sarcoplasm extracts that were not treated with TCA. Both methods meet the requirements I described above, and a study to should be conducted to compare and evaluate the precision and recovery rate of both methods to validate their practicality for future free calcium research.

Second, a poor relationship was found between lipid oxidation in muscle tissue and proteolysis in these dissertation studies. Data from the first and third manuscripts strongly suggested that membrane oxidation has an effect on calcium release, and likely, the increase in free calcium will further stimulate calpain activity and improve meat tenderness. However, the conventional thiobarbituric acid reactive substances (TBARS) method using a spectrophotometer is relatively insensitive and not capable to quantify samples in small volume. A sensitive, simple, and reliable approach that can detect lipid oxidation in extremely small sample volumes needs to be developed for direct measurement of SR membrane oxidative status. Yagi (1987) developed a sensitive, simple, and reliable fluorometric method that can detect lipid oxidation products as low as 0.1 nM in human plasma. Jo and Ahn (1998) further modified the method to work in meat. A study applying and validating a way to measure lipid oxidation products of SR membrane using the modified Yagi (1987) method is pertinent for any future study based on organelle membrane oxidation.

Third, the effects of diets on sarcoplasmic reticulum (SR) fatty acid, phospholipid and total lipid profiles have been established in this dissertation, but the true effect of

oxidation on these profiles has not been well documented. Álvarez et al. (2008) documented the modifications in fatty acid composition of lamb meat during storage in high-oxygen modified atmosphere packaging, and Ji and Takahashi (2006) found phospholipid content of SR membrane decreased during the aging of pork and beef. Many other studies have also indicated a relationship between fatty acid oxidation and phospholipid degradation (Brasitus et al., 1985; Deaver Jr et al., 1986; Thi-Dinh et al., 1990), but no clear pattern has been established. It has been suggested that phospholipases increase in activity to remove esterified polyunsaturated fatty acids when lipid oxidation occurs, thus resulting in phospholipid liberation (Leshem, 1987; Mead et al., 1980). Induced oxidation (high-oxygen modified atmosphere packaging) is a great way to generate extremely oxidized samples. Further evaluation of dietary treatments on organelle fatty acid, phospholipid and total lipid profiles from fresh and oxidized samples can truly shed light on the pattern of fatty acid oxidation and phospholipid degradation.

Finally, this research has shown that high PUFA in the diet can increase the proportion of phosphatidylcholine (PC) or the conversion of phosphatidylethanolamine (PE) to PC in SR and such a shift has an effect on early postmortem proteolysis. However, what is the mechanism behind it? Fu et al. (2011) demonstrated that the conversion of PE to PC in the endoplasmic reticulum (ER) is closely linked to ER oxidative stress. In the mitochondria, changes in the mechanism responsible for regulation of mitochondrial functions can lead to increases electron leakage from the electron transport chain, which can cause a potential reduction in ATP synthesis (Grubbs et al., 2013). Chance et al. (1979) pointed out that such electron leakage leads to the production of reactive oxygen species (ROS), and when leakage is excessive, oxidative

damage to DNA, lipids (Yu, 1994) and proteins (Dröge, 2002) may occur. I hypothesize that a diet high in PUFA, such as WDGS, increases overall oxidative stress in cattle, which causes an increased production of ROS in the mitochondria. An excessive amount of ROS or an inadequate amount of antioxidants can lead to SR protein damage pre-harvest. Oxidized SR proteins are destined to the ubiquitination pathway and degraded by the proteasome (Mehlhase and Grune, 2002); the elevated conversion of PE to PC in the SR membrane likely occurred because less functional sarco/endoplasmic reticulum calcium ATPase (SERCA) are available for interaction, which makes the PE to PC conversion a great indicator for SR oxidative stress. The SR functionality is closely linked to the integrity of SR proteins. The oxidized SR SERCA are less functional even when there are ample supplies of ATP left, leaving plenty of calcium ions in the sarcoplasm, which in combination with higher temperature and pH, eventually leads to increased early calpain activity. To explore this hypothesis, the following questions have to be answered.

How to quantify oxidative stress in SR and mitochondria?

When does the SR protein oxidation start to occur?

How does feeding diets high in PUFA affect SR protein oxidation?

How can we correlate SR protein oxidation, pre-rigor free calcium content and subsequent proteolysis?

To take this concept even a step further, one should look into the field of proteomics to identify changes in specific proteins in relationship to this proposed SR oxidative stress. It has been shown that oxidized RyR1 can stimulate calcium release by forming a disulfide bond to maintain the channel in the open state (Abramson and

Salama, 1989). Perhaps, the oxidation of luminal proteins, especially calsequestrin, resulted in adverse effects on SR storage ability, and oxidation of SERCA may also lead to calcium accumulation pre-rigor and early post-rigor. A study incorporating post-mortem calcium release, SR membrane lipid and protein oxidation, proteolysis and the change of SR membrane lipid and protein profile will answer many of the questions raised from the hypothesis of this study.

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# Appendix I

Composition of Finishing Diets on a Dry Matter (DM) Basis

Composition of finishing diets for manuscript 1.

| | 50% WDGS | Corn-only |
|---|---|---|
| Ingredients, % of DM | | |
| Dry rolled corn | 16.5 | 41.5 |
| High moisture corn | 16.5 | 41.5 |
| Wet distillers grains plus solubles | 50 | 0 |
| Corn silage | 12 | 12 |
| Supplement[1] | 5 | 5 |

[1]Formulated to contain 380 mg/hd/d of Rumensin and 90 mg/hd/d of Tylan.

Composition of finishing diets for manuscript 2 and 3.

| | 30% WDGS | Corn-only |
|---|---|---|
| Ingredients, % of DM | | |
| Dry rolled corn | 26.5 | 41.5 |
| High moisture corn | 26.5 | 41.5 |
| Wet distillers grains plus solubles | 30 | 0 |
| Corn silage | 12 | 12 |
| Supplement[1] | 5 | 5 |

[1]Formulated to contain 380 mg/head/day of Rumensin and 90mg/head/day of Tylan with or without vitamin E (E) at 450 mg (1,000 IU)/hd/d; Agrado Plus (AG) at 3 g/hd/d (215 mg/kg of feed); a combination of 225 mg (500 IU)/hd/d of E and 3 g/hd/d (215 mg/kg of feed) of AG.

## Appendix II

Sarcomere Length of Powdered Meat Samples

(Dolazza and Lorenzen 2014; Cross et al. 1981)

1. Spread the powdered meat sample very lightly (just a few specks) on the microscope slide.
2. Place a drop of 0.25M sucrose solution on the slide and cover with a coverslip.
3. Place the slide on the stage of the laser stand (the distance between the slide and the base of the laser stand should be set to 100 mm).
4. Place a piece of paper at the base of the laser stand.
5. Move the slide back and forth through the laser light until a diffraction pattern is observed (Figure 1).

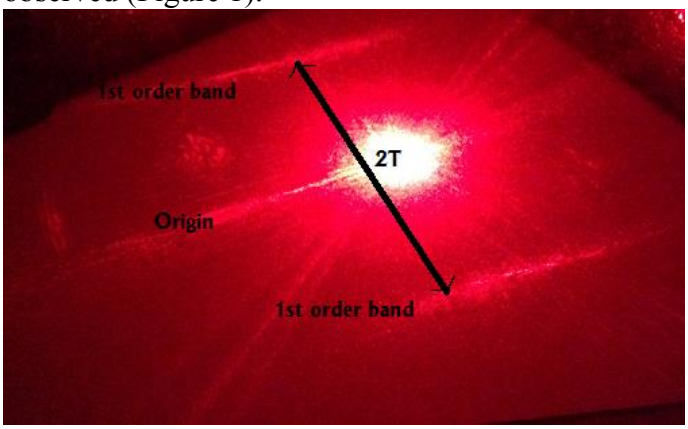


Figure 1. A projected sarcomere diffraction pattern on the paper

6. Mark the original and the two diffraction bands for 5 different sarcomeres from the sample.
7. Measure the distances between the two diffraction bands and calculate sarcomere length using the following equation:

$$\text{Sarcomere length } (\mu m) = \frac{0.6328\ X\ D\sqrt{(\frac{T}{D})^2+1}}{T}$$

D= distance in mm from slide to the base of laser stand.

T = ½ of the distance in mm from one first order band to the other first order band.

8. Use the average the 5 sarcomere lengths to determine the sarcomere length of the sample.

References:

Cross, H., R. West, and T. Dutson. 1981. Comparison of methods for measuring sarcomere length in beef *semitendinosus* muscle. Meat Sci. 5: 261-266.

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**Appendix III**

Myofibrillar Protein Isolation
(Pietrzak et al., 1997)

1. Weigh 3 g of powdered meat sample into a 50 mL plastic centrifuge tube.
2. Add 15 mL ice-cold rigor buffer (0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 10
3. mM $K_2HPO_4$; pH 7.4) and homogenize using the Polytron (Kinematica CH-6010, Switzerland) at very low speed for 15 s.
4. Filter the homogenate through double-layered cheese cloth to remove connective tissue and fats into a new 50 mL plastic centrifuge tube.
5. Pipette 1.4 mL homogenate into an eppendorf tube (2 mL safe-lock tubes; Eppendorf, Hamburg, Germany).
6. Centrifuge at 4000 × g for 5 min.
7. Decant the supernatant and dismantle the pellet using a cleaned spatula after resuspending in 1 mL of ice-cold rigor buffer.
8. Vortex the mixture for 10 s and centrifuge for 5 min at 4000 × g.
9. Repeat step 6 and 7 two more times to remove myoglobin as much as possible (until the supernatant is clear and free of myoglobin).
10. Decant the supernatant and remove any leftover supernatant using a pasture pipette.
11. Re-suspend the pellet in 1 mL of extraction buffer (0.1M Tris-HCl, 1.25 mM EDTA, 5% SDS).
12. Vortex thoroughly after dismantling the pellet.
13. Keep the sample in room temperature for 5 min.
14. Centrifuge for 5 min at 4000 × g.
15. Transfer 100 μL of the supernatant to a new eppendorf tube for determination of protein concentration.

Reference:

Pietrzak, M., M. L. Greaser, and A. A. Sosnicki. 1997. Effect of rapid rigor mortis processes on protein functionality in pectoralis major muscle of domestic turkeys. J. Anim. Sci. 75: 2106-2116.

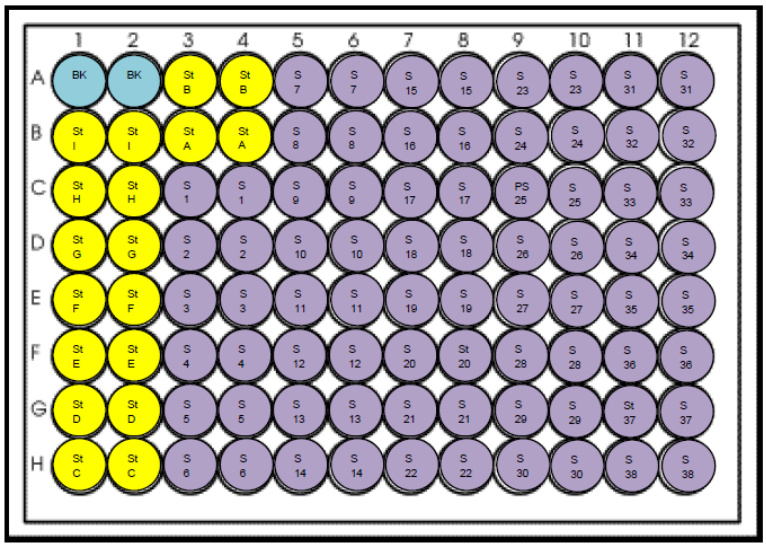
# Appendix IV

Determine Protein Concentration
(Pierce BCA protein assay kit; Pierce Biotechnology, Rockford, IL)

1. Add 0.9 mL of extraction buffer to the new eppendorf tube with 100 µL of myofibrillar protein stock samples and vortex thoroughly.
2. Prepare a concentration series (20 - 2000 µg/mL) of bovine serum albumin (BSA) in the kit as following:

| Vial | Diluent (µL) | BSA (µL) | BSA concentration (µg) |
|---|---|---|---|
| A | 0 | 300 of stock | 2000 |
| B | 125 | 375 of stock | 1500 |
| C | 325 | 325 of stock | 1000 |
| D | 175 | 175 of vial B dilution | 750 |
| E | 325 | 325 of vial C dilution | 500 |
| F | 325 | 325 of vial E dilution | 250 |
| G | 325 | 325 of vial F dilution | 125 |
| H | 400 | 100 of vial G dilution | 25 |
| I | 400 | 0 | 0 = blank |

3. Place 25 µL of BSA standards and the diluted myofibrillar protein samples on a 96 wells microplate as following:




BK – Blank
St – BSA standards
S – Samples

4. Add 200 µL BCA working reagents (50:1, Reagent A:Reagent B) to respective wells in the microplate.
5. Mix protein samples and BCA working reagents thoroughly on a plate shaker for 30 seconds.
6. Incubate the microplate at 37ºC for 30 min and cool to the room temperature.

7. Read absorbance at 562 nm with a microplate spectrophotometer (Model Epoch, Biotek, Winooski, VT).
8. Protein concentrations are expressed as µg/mL.
9. Remove another 100 µL of protein stock to a new eppendorf tube.
10. Dilute the protein to 2 mg/mL with extraction buffer.
11. Transfer 25 µL of the diluted protein sample to a new eppendorf tube.
12. Add 25 µL of 2 x Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) to the diluted protein sample in the new tube (The protein concentration is now at 1 g/mL).
13. Heat the tube on heating block at 95ºC for 5 min.
14. Store prepared sample in -80$^{o}$C until use.

**Appendix V**

Gel Electrophoresis
(Mini-PROTEAN Tetra Cell; Bio-Rad)

1. Remove the Mini-PROTEAN TGX Precast Gel 4-20% from the package.
2. Pull the tape off the bottom of the gel, and remove the comb.
3. Place it into the electrode assembly with short plate inward.
4. Place the second gel on the other end of the assembly.
5. Lock the two gels in place.
6. Place the assembly into the tank, make sure to match the red banana plug with the red oval and the black plug with black oval.
7. Fill the chamber with ice cold 1x Tris/Glycine/SDS running buffer (Bio-rad) until the short plate is completely covered.
8. Add running buffer to the outer tank to either 2 or 4 gel line depend on how many gels you are running.
9. Load 10 µL Bio-Rad Kaleidoscope Pre-stained standards in the first well.
10. Load 5 µL (5 µg of protein) of prepared sample into each of the rest of the well.
11. Place the lid on the mini tank aligning color coded banana plugs and jacks.
12. Insert the electrode leads into a Bio-Rad high current power supply (PowerPac HC power supply; Bio-Rad).
13. Set voltage to 200 V.
14. Run until the tracking dye in the Leamli buffer of each sample reaches the finishing line (About 40 min).
15. After electrophoresis is complete, turn off the power supply and disconnect the electrical leads.
16. Remove gel sandwich from the gel assembly.
17. Remove the gel by gently separating the two plates with the opening key provided in the precast gel package.
18. Square the gel by trimming the wells and the bottom using a guillotine blade.

**Appendix VI**

Western Blotting
(Mini Trans Blot Cell; Bio-Rad)

1. Equilibrate the gel in transfer buffer (25 mM tris-base, 192 mM glycine, 20% methanol, 0.1% SDS at pH 9.2) for 20 min.
2. Soak precut filter papers (Bio-Rad) and fiber pads in transfer buffer for 5 min.
3. Wet Polyvinylidene fluoride (PVDF) membranes (IPFL20200; Millipore, Bedford, MA) in methanol for 15 seconds and rinse briefly in deionized water before soaking in transfer buffer.
4. Prepare the gel sandwich as following:

-Place the cassette with the black side (anode) down in transfer buffer in the cassette assembly box.
-Place one pre-wetted fiber pad on the black side of the cassette.
-Place a filter paper on the fiber pad.
-Place the equilibrated gel on the filter paper (roll out all bubbles).
-Place the pre-wetted membrane on the gel (roll out all bubbles).
-Place the other filter paper (roll out all bubbles) and fiber pad respectively.
-Close the cassette firmly without moving the gel and filter paper sandwich and lock the cassette with the white latch).

5. Place 2 prepared cassettes in module.
6. Place the module in the tank.
7. Place an ice pack in the tank.
8. Fill the tank with ice-cold transfer buffer to the blotting line on the tank.
9. Set the voltage at 100 V for 60 min.
10. Remove the membrane from the sandwich.
11. Place the membrane in a container with 10 mL Odyssey Blocking Buffer (LI-COR, Lincoln NE) for 120 min in room temperature on a rocking platform.

A. Primary Antibody Preparation and Incubation

1. Dilute primary antibodies (Anti-Troponin-T JLT-12; Sigma-Aldrich, St. Louis, MO) 1:10,000 with Odyssey Blocking Buffer.
2. Incubate membranes in 10 mL of diluted primary antibody for 1 h in room temperature on a rocking platform.
3. Pour off primary antibody solution.
4. Wash membrane 3 times for 10 min each with 15 mL of 1X TBST (Tris buffered Saline+0.1% Tween 20) on a rocking platform.

B. Secondary Antibody Preparation and Incubation

1. Reconstitute contents in the IRDye 680LT Conjugated Goat Anti-Mouse IgG1 (LI-COR) with 0.5 mL of deionized distilled water. Mix gently by inverting and allow rehydrating for at least 30 min before use. (Centrifuge product if solution is not completely clear after standing at room temperature. After reconstitution, it is stable for up to 3 month at 4ºC without exposure to direct light.
2. Dilute the secondary antibody 1: 10,000 with Odyssey Blocking Buffer.
3. Incubate membranes in 10 mL of the diluted secondary antibody for 60 min in room temperature on a rocking platform (light sensitive, perform in foil covered box from this step on).
4. Pour off secondary antibody solution.
5. Wash membranes 3 times for 10 min each with 15 mL of 1X TBST on rocking platform.
6. Wash membrane with 15 mL of 1X TBS for 5 min to remove any residual Tween 20.
7. Protect membrane from light during incubation, washing and storage.

C. Membrane Imaging

1. Membrane can be imaged wet or dry.
2. Measure desired bands using Odyssey Infrared Imaging System (LI-COR) at 700 nm channel as integrated intensity (K. pixels).
3. Imaging conditions:
Resolution: 169 nm
Quality: low
Focus offset: 0 mm
Channels: 700 nm
Intensity: 3.0 or 3.5
Image size: x-10 y-7

**Appendix VII**

SR Membrane Extraction
(Hemmings, 2001)

1. Weighed 10 g of powdered meat sample to a 100 ml plastic beaker.
2. Add 35 ml of ice cold homogenization buffer : 10mM $NaHCO_3$ (0.849g/L), 2 mM sodium azide (0.13g/L), 10 mM Tris-Cl (1.21g/L), pH 7.5.
3. Homogenize the minced muscle on ice with Polytron homogenizer (large probe, setting 6) on ice, using 3 x 15 s bursts, with a 30 s rest between bursts (Move the pestle up and down to ensure total homogenization).
4. Filter the homogenate with two layers of cheesecloth into a 50 ml plastic centrifuge tube.
5. Centrifuge at 2,000 x g for 10 min at 4$^{o}$C.
6. Collect and retain the supernatant.
7. Add 10 ml of homogenization buffer and re-suspend the pellet.
8. Centrifuge at 2,000 x g for 10 min at 4$^{o}$C.
9. Collect the supernatant and combine with the retained supernatant in a 50 mL Oak Ridge high-speed centrifuge tube (Thermo Scientific, Rockford, IL).
10. Centrifuge the supernatants at 10,000 x g for 30 min at 4$^{o}$C using a superspeed centrifuge (Sorvall RC5B, Thermo Scientific).
11. Transfer the supernatant to a 50 ml plastic centrifuge tube.
12. Add 4.5 g KCl per 100 ml supernatant.
13. Place the tubes with the supernatant on a shaker and shake for 30 min on ice.
14. Transfer the supernatant to a thick wall ultracentrifuge tube (25 x 89 mm; Beckman Coluter, Brea, CA)).
15. Centrifuge at 100,000 x g for 60 min at 4$^{o}$C (L7-65 Ultracentrifuge with a SW28 rotor; Beckman Coluter)
16. Discard the supernatant, and use a transfer pipet to remove any remaining supernatant.
17. Add 1 ml of 10 mM Tris-Cl and suspend the pellet in the solution.
18. Transfer the suspended pellet to a 13x100 mm screw cap glass tube.
19. Store the sample in -80 $^{o}$C freezer.

Reference:

Hemmings, S. J. 2001. New methods for the isolation of skeletal muscle sarcolemma and sarcoplasmic reticulum allowing a comparison between the mammalian and amphibian beta(2)-adrenergic receptors and calcium pumps. Cell Biochem. Funct. 19: 133-141.

**Appendix VIII**

SR Membrane Lipid Extraction
(Bligh and Dyer, 1959)

1. Add 3.75 mL of 1:2 chloroform:methanol (v/v) to the 13x100 mm glass tube with the SR homogenate.
2. Vortex for 5 s and mix on shaker for 20 min.
3. Keep the mixture at $4^{o}$C overnight.
4. The next morning, add 1.25 ml chloroform and mix on shaker for 1 min.
5. Add 1.25 ml water and mix on shaker for another minute.
6. Filter homogenate through Whatman #2 filter paper into another 13 x 100 mm screw cap tube.
7. Centrifuge samples at 1,000 x g for 5 min. Following centrifugation, aspirate off the aqueous phase (top layer).
8. Evaporate to dryness under nitrogen at 60°C.
9. Continue on for either fatty acid analysis or lipid profile analysis.

Reference:

Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

**Appendix IX**

SR Fatty Acids

Gas Chromatography (GC) (Metcalfe et al., 1966; Morrison and Smith, 1964)

1. Add 0.5 mL of 0.5 M NaOH in methanol to each dried SR membrane lipid sample from the SR membrane lipid extraction. Vortex for 5 sec.
2. Heat in the oven for 5 min at 100°C.
3. Add 0.5 mL of boron trifluoride in 14% methanol. Vortex for 5 sec.
4. Heat in the oven for another 5 min at 100°C.
5. Add 1 mL of a saturated salt solution and 1 mL of hexane. Vortex for 5 sec.
6. Centrifuge samples at 1,000 x g for 5 min. Following centrifugation, remove hexane layer (top layer) **making sure not to disrupt the aqueous phase** (lower layer) and place in GC vial.
7. Concentrate the lipid by evaporating the hexane layer to dryness under nitrogen at 60°C in the GC vial.
8. Add 100 µL of hexane and transfer the content to a 100 µL spring bottom vial inserts.
9. Place the insert inside the GC vial and purge with nitrogen, cap and crimp cap, and store at -80°C until sample is ready to be read on the GC.

**GC Settings:**
**Column -** Chrompack CP-Sil 88 (0.25 mm x 100 m)
**Injector Temp -** 270°C
**Detector Temp -** 300°C
**Head Pressure -** 40 psi
**Flow Rate -** 1.0 mL/min

**Temperature Program -** Start at 140°C and hold for 10 min. Following 10 min, raise temperature 2°C/min until temperature reaches 220°C. Hold for 20 min at 220°C.

References:

Metcalfe, L. D., A. A. Schmitz, and J. R. Pelka. 1966. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chem. 38: 514-515.

Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride--methanol. J. Lipid Res. 5: 600-608.

**Appendix X**

SR Phospholipids and Neutral Lipids
1D Thin Layer Chromatography (TLC) (Leray et al., 1987)

1. Add 30 µl of (2% methanol and 1 % distilled deionized water in chloroform) to each dried SR membrane lipid sample from the SR membrane lipid extraction.
2. TLC plates (Whatman K5, Clifton, NJ) are pre-washed by migration up to 1 cm from the top in chloroform/methanol (1:1, v/v).
3. Air-dry plate completely in fume hood.
4. Plates are thoroughly wetted with 2.3% boric acid solution in ethanol.
5. Drained in fume hood till the TLC plate is completely free of wet spot.
6. Dry the TLC plate for 15 min at 100°C in an oven.
7. Draw reference starting lines using a pencil.
8. Spread 20 µL of lipid standards at the first column, and spread all remaining lipid from each sample on its designated column (~6 samples per TLC plate). Be sure to remove any air bubble.
9. Place the prepared TLC plate in about 100 mL of solvent (Chloroform/ethanol/water/triethylamine, 30:35:7:35 v/v/v/v) in a TLC tank till migration to 1 cm from the top (about 2-3 hrs).
10. Dry in fume hood till the plate is completely dry.
11. Spray the whole plate wet with dye (10% cupric sulfate in 8% phosphoric acid).
12. Dry in fume hood till the plate is completely dry (30 – 60 min).
13. Place TLC plates in oven at 180°C until the lipid spots show (Check every 5 min to avoid over-burning the plates, time varies from 5 - 30 min).
14. Analyze the plate using Quantity One 1D image analysis software (Bio-Rad, Hercules, CA).
15. Each phospholipid or neutral lipid was measured as a percentage of total phospholipids or neutral lipid in one lane.
16. For total lipid profiling, all phospholipids and all neutral lipids from one lane were combined as one class. Each lipid class was measured as a percentage of total lipids on one specific lane.

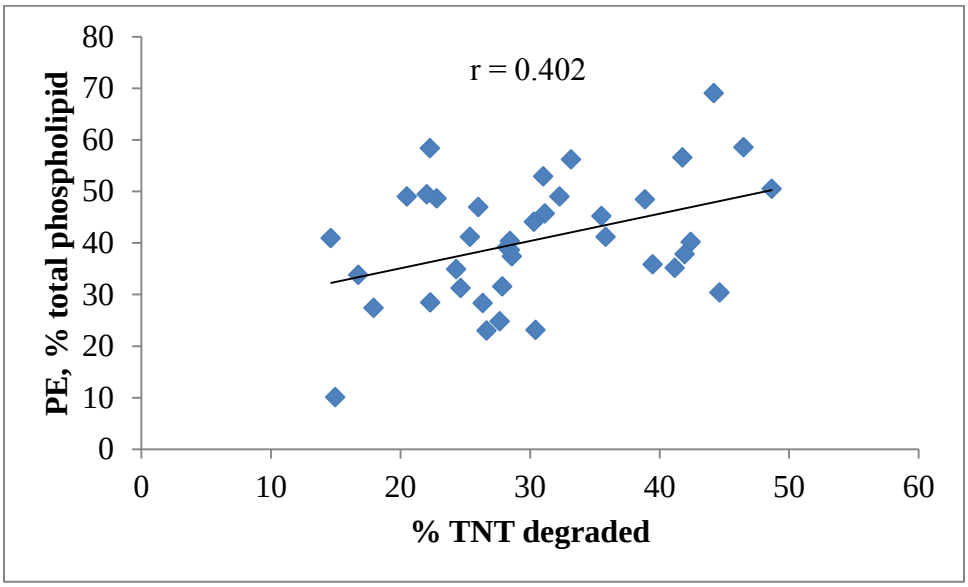
Reference:

Leray, C., X. Pelletier, S. Hemmendinger, and J. P. Cazenave. 1987. Thin-layer chromatography of human platelet phospholipids with fatty acid analysis. J. Chromatogr. 420: 411-416.

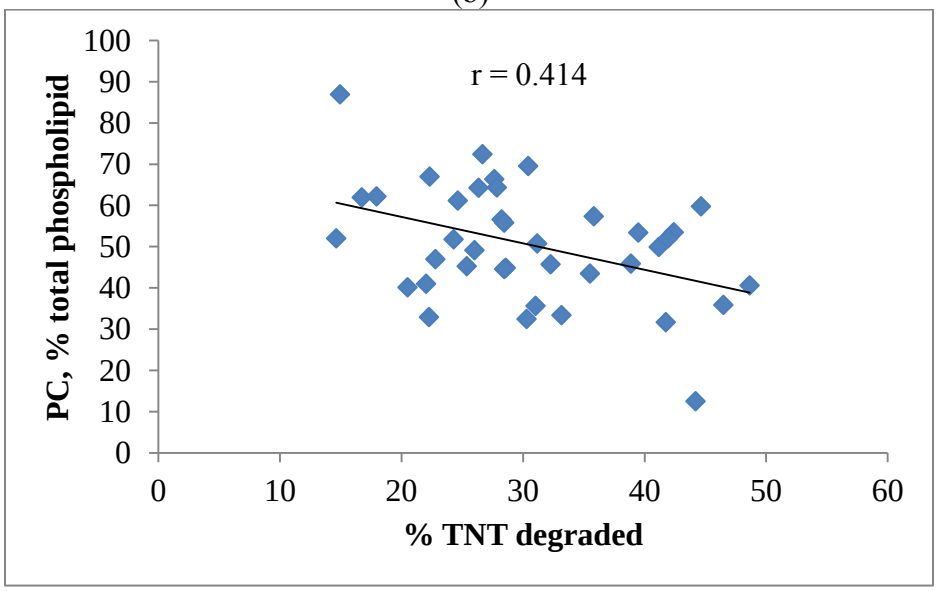
## Appendix XI

Relationship of the troponin-T (TNT) degradation (muscle aged 2 d) with a) phosphatidylethanolamine (PE) and b) phosphatidylcholine (PC) in total sarcoplasmic reticulum (SR) membrane phospholipid.

(a)




(b)



## Appendix XII

Warner-Bratzler Shear Force, kg of strip loins (*Longissimus lumborum*) from steers fed corn only or corn with 30% wet distillers grains plus solubles (WDGS) supplemented with or without vitamin E (E) or Agrado (AG) or a combination of E and AG (Manuscript 3).

| | Dietary treatments | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Retail display, d | Corn | Corn+E | Corn+AG | Corn+E+AG | 30%WDGS | WDGS+E | WDGS+AG | WDGS+E+AG | SEM | *P*-value |
| 2 d aged | | | | | | | | | 0.2 | 0.65 |
| 0 | 4.66 | 4.05 | 3.89 | 4.09 | 4.30 | 4.30 | 3.99 | 4.01 | | |
| 7 | 3.48 | 3.32 | 3.29 | 3.39 | 3.50 | 3.19 | 3.08 | 3.00 | | |
| 7 d aged | | | | | | | | | | |
| 0 | 3.05 | 3.51 | 3.54 | 3.12 | 3.69 | 3.35 | 2.95 | 3.12 | | |
| 7 | 3.13 | 3.15 | 3.13 | 3.07 | 3.12 | 3.08 | 3.06 | 3.07 | | |
| 14 d aged | | | | | | | | | | |
| 0 | 3.01 | 3.04 | 2.88 | 2.87 | 2.95 | 2.95 | 2.79 | 2.93 | | |
| 7 | 2.90 | 2.99 | 2.63 | 2.79 | 2.73 | 2.58 | 2.65 | 2.90 | | |